UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia Química

CARLA FERREIRA DOS SANTOS VIEIRA

DEVELOPMENT OF AN ADVANCED FERMENTATION TECHNOLOGY TO PRODUCE N-BUTANOL, ISOPROPANOL, AND ETHANOL FROM SUGARCANE BAGASSE

DESENVOLVIMENTO DE TECNOLOGIA AVANÇADA DE FERMENTAÇÃO PARA A PRODUÇÃO DE N- BUTANOL, ISOPROPANOL E ETANOL A PARTIR DE BAGAÇO DE CANA-DE-AÇÚCAR

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Tese apresentada à Faculdade de Engenharia Química da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Engenharia Química.
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Supervisor/Orientador: Prof. Dr. Adriano Pinto Mariano
Co-supervisor/Coorientador: Prof. Dr. Francisco Maugeri Filho

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In memory of my dear father, Prof. Manoel Vieira.

“Ser graduado pela escola superior da vida é muito mais difícil do que ser graduado por outras escolas superiores reconhecidas pelo MEC.”

“É utópico imaginar uma sociedade justa. É racional lutarmos por uma sociedade menos injusta.”

Manoel Vieira

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RESUMO

O biobutanol é convencionalmente produzido através da fermentação ABE (acetona-butanol-etanol). No entanto, a alta corrosividade e as baixas propriedades funcionais da acetona tornam o processo menos atrativo para a produção de butanol como biocombustível. Alternativamente, a produção de biobutanol via fermentação IBE (isopropanol-butanol-etanol) diminui esses riscos de mercado, uma vez que essa mistura pode ser utilizada diretamente como biocombustível ou como aditivo na gasolina. Contudo, limitações técnicas presentes na fermentação ABE, como baixa produtividade, alta inibição pelo produto, e sensibilidade aos inibidores fermentativos oriundos do processo de segunda geração, são ainda mais intensos na fermentação IBE. Portanto, propusemos nessa tese de doutorado, a integração, em um mesmo tanque fermentativo, de um sistema de imobilização celular, que aumenta o número de células no reator, à tecnologia de extração a vácuo, que permite a recuperação *in-situ* dos produtos de fermentação, diminuindo a inibição pelo produto. A tecnologia de impressão 3D foi utilizada para construir o sistema de imobilização celular, composto por uma estrutura tipo gaiola que mantinha o bagaço em contato com o meio de cultura ao longo de todo o processo fermentativo. Propusemos ainda, uma estratégia que permitiu a produção de IBE a partir de hidrolisados de bagaço de cana-de-açúcar através da adição de melaço. Com a utilização do sistema de imobilização celular proposto nesse trabalho, conseguimos realizar a fermentação IBE em 5 bateladas consecutivas (138 horas) em meio sintético. No entanto, a conversão de glicose e a produtividade foram limitadas a 37 % e 0,21 g IBE/L·h, respectivamente. Ao acoplar a tecnologia de extração a vácuo, conseguimos conduzir uma fermentação IBE em modo batelada-repetida por 209 horas, em que a conversão de glicose e a produtividade aumentaram para 66 % e 0,28 g IBE/L·h, respectivamente. Ao final do processo, obtivemos um condensado contendo 29 g/L de butanol, concentração mais elevada que as atingidas em reatores simples, o que geraria economia de energia no processo de separação. Em paralelo, mostramos que, apesar da presença de compostos inibidores do processo fermentativo, a utilização de hidrolisados lignocelulósicos do bagaço de cana é possível para a produção de IBE, especialmente quando o melaço é acrescentado como suplemento. Em meio contendo um total de açúcares de 35 g/L, a glicose foi completamente consumida e a sacarose, xilose e o ácido lático foram consumidos em 38%, 31%, e 70%, respectivamente. Nós agradecemos à Fundação de Amparo à Pesquisa do Estado de São Paulo (Processos 2015/20630-4; 2016/23042-9; 2017/07390-0, e 2018/23983-3) pelo apoio financeiro.

Palavras-chave: Biocombustível, butanol, batelada-repetida, imobilização celular, *Clostridium beijerinckii*, fermentação a vácuo.

ABSTRACT

Biobutanol is conventionally produced through ABE (acetone-butanol-ethanol) fermentation. However, acetone's corrosivity and poor fuel properties decrease attractiveness of this process for butanol production as biofuel. Alternatively, the production of biobutanol through IBE (isopropanol-butanol-ethanol) fermentation decreases these market risks since the IBE mixture can be used directly as fuel or as gasoline additive. However, technical limitations commonly found in ABE fermentation, i.e. low productivities, high product inhibition, and high sensitivity towards fermentation inhibitors from second generation processes, are even more accentuated in IBE fermentation. Thus, this thesis proposed the integration, in the same fermentation vessel, of a cell immobilization system, which increases the number of cells inside the bioreactor, and the vacuum extraction technology, that promotes *in-situ* product recovery, decreasing product inhibition. 3D printing technology was used to build the immobilization system, composed by a cage-like prototype that maintained the sugarcane bagasse in contact with the fermentation medium along the process. This thesis also proposed a strategy to enable IBE production from sugarcane bagasse hydrolysates using molasses as supplement. The immobilization system allowed the performance of five consecutive batches (138 hours) in synthetic medium. However, glucose conversion and IBE productivity were limited to 37 % and 0.21 g/L·h, respectively. Coupling vacuum technology to the system allowed the conduction of 209 hours of repeated-batch process; glucose conversion and IBE productivity increased to 66 %, 0.28 g/L·h, respectively. At the end of the fermentation, we obtained a condensate with 29 g butanol/L; this higher butanol concentration compared to concentrations achieved inside the bioreactor can decrease energy consumption during separation process. Parallelly, this thesis showed that, despite the presence of fermentation inhibitory compounds, the use of sugarcane bagasse lignocellulosic hydrolysates is feasible for IBE production, specially when molasses is added as supplement. Glucose was exhausted and sucrose, xylose, and lactic acid consumption were 38 %, 31 %, and 69 %, respectively. We thank the São Paulo Research Foundation (FAPESP) for the financial support (Grant numbers 2015/20630-4; 2016/23042-9; 2017/07390-0, and 2018/23983-3).

Keywords: Biofuel, butanol, repeated-batch, cell immobilization, *Clostridium beijerinckii*, vacuum fermentation.

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1. INTRODUCTION

1.1. Context

Biobutanol is traditionally produced through ABE (acetone-butanol-ethanol) fermentation. However, acetone, which accounts for 20 – 30 % of ABE production, has poor fuel properties and is corrosive to engines, which prevent the mixture to be used directly as biofuel (Li et al., 2016). For this reason, if butanol production achieves the scale of billion liters in response to automotive fuel market demands, we can expect an oversupply of acetone (Mariano et al., 2013b).

Alternatively, some *Clostridium beijerinckii* strains can convert acetone to isopropanol, performing the IBE (isopropanol-butanol-ethanol) fermentation (Vieira et al., 2019). The fuel properties of the IBE alcohol mixture allow its utilization directly as fuel or as gasoline additive, decreasing market risks related to acetone. Moreover, since IBE separation is not necessary, process energy efficiency gains are expected (Li et al., 2016). However, IBE fermentation technical limitations persist. High product inhibition, high fermentation time, low productivity, low cell density, and high sensitivity against second-generation fermentation inhibitors (e.g. organic acids, phenolics, and furans) are important drawbacks of the IBE fermentation (Vieira et al., 2019).

High product inhibition is one of the most studied bottlenecks of the ABE fermentation (Kolesinska et al., 2019) and, unfortunately, IBE producing Clostridia are even more sensitive to butanol, leading to less efficient fermentation performances (Vieira et al., 2019; Vieira et al., 2020) . This problem is responsible for low process productivities, and incomplete sugar conversion, which increases substrate costs. Therefore, besides metabolic engineering (reviewed by Vieira et al., 2019), in-situ product recovery has been an important strategy to reduce product inhibition in IBE fermentation. This technology comprises product extraction while it is produced,

allowing a more complete substrate utilization; thus, increased productivity (due to the possibility of using higher substrate concentrations), and decreased energy consumption in distillation step (a more concentrated product stream is obtained) are also great advantages (Mariano & Maciel Filho, 2012).

Adsorption (Groot & Luyben, 1986), pervaporation (van der Heiiden & Groot, 1989), gas-striping (Matsumara et al., 1992; Vrije et al., 2013; Pyrgaskis et al., 2016;), and liquid-liquid extraction (Lee et al., 2012; Zhang et al., 2017; Xin et al., 2017; Pérez-Bibbins et al., 2018; Survase et al., 2019) are on-line product recovery techniques already tested for IBE fermentation (a summary of the results can be found in Vieira et al., 2019). However, although vacuum fermentation has already showed efficiency in butanol recovery during ABE fermentation (Mariano et al., 2008; Mariano et al., 2011, 2012a,b), it had not been tested yet in the IBE process.

Originally, this technology was developed for ethanol fermentation in the 1970s, but Mariano et al. (2008) demonstrated its applicability for butanol production through ABE fermentation. Vacuum extraction does not comprise membranes or gas stripping. Therefore, it is not susceptible to clogging by lignocellulose fibers, which avoids the necessity for equipment for removing insoluble solids before the fermentation. However, even with all advantages offered by this technology, in a single-batch ABE fermentation equipped with the vacuum system, productivity gains compared to control experiment were limited to 8 – 30 %, depending on the frequency of vacuum sessions applied during the fermentation (continuous or intermittently) (Mariano et al. 2011).

Nevertheless, vacuum extraction offers high butanol removal rates (between 1.4 and 16.6 g/L·h), approximately 10 times “faster” than gas stripping (Mariano et al., 2011), which makes it especially suitable for high cell concentration systems offered by cell immobilization. Indeed, attempting to increase productivity gains offered by in-situ

product recovery, previous studies associated cell immobilization with product extraction. Sweet sorghum bagasse for example, was already used as cell holding material in an ABE fermentation system equipped with gas stripping (Cai et al., 2015). Sugarcane bagasse and liquid–liquid extraction were also tested together for ABE production (Bankar et al., 2012).

Besides the increase of cell density and fermentation productivity, the use of a cell carrier also allows the reutilization of the grown cells in repeated-batch processes, eliminating the necessity of inoculum preparation and reducing lag-phases, which also contribute to productivity gains (Koleinska et al., 2019). However, the use of a lignocellulose material e.g. sugarcane bagasse as cell carrier in repeated-batch fermentation can be difficult due to tubes clogging during the bioreactor draining. Therefore, a structure to trap the sugarcane bagasse as cell carrier, but that also allows its contact with the fermentation broth, would be necessary, creating a fermentation system similar to a fixed bed bioreactor.

Attempting to design and characterize a fixed bed bioreactor, Kilonzo et al., 2010 studied an air-lift fibrous bed bioreactor where woven cotton was used as cell carrier. Researchers showed that spiral-wound fibrous bed can offer better homogenization and solid-to-liquid mass transfer performance. In our immobilized system, where sugarcane bagasse would be used as cell carrier, fermentation products would be accumulated between the biofilms adhered to the bagasse; the motion of bubbles formed due to broth evaporation during vacuum would be similar to motion of gas bubbles present in an air-lift bioreactor. Therefore, the use of a cage-like structure similar to the spiral-wound fibrous bed designed by Kilonzo et al., 2010, could improve homogenization and mass transfer, and consequently, product recovery performance by the vacuum system.

Besides all technology advances related to the fermentation system, another

important component of the IBE production process is the substrate. Since most published works comprise fermentation systems and metabolic engineering, glucose has been the most used carbon source for IBE production (Vieira et al., 2019). Although, nowadays, the use of second-generation feedstock is mandatory due to climate concerns. However, since these substrates are more complex, their use as carbon source for IBE production is challenging, even though IBE-producing clostridia can metabolize several types of lignocellulosic sugars, including xylose (Survase et al., 2013).

Besides, biomass pretreatment harsh conditions are known to generate compounds (e.g., furans, organic acids, and phenolics) that decrease fermentation yields and productivities (van der Pol et al., 2014), and IBE producing Clostridia are especially sensitive (Bankar et al., 2014; Survase et al., 2019). Additionally, literature suggests that enzymatic hydrolysis conditions (45–50°C, pH 4.8–5.0) combined with long batch time (up to five days) and improper storage allow the cellulose hydrolysate to be more vulnerable to microbial contamination. Consequently, cellulose hydrolysate containing fermentation inhibitors, such as lactic and acetic acid, can also challenge IBE fermentation (Schell et al., 2006; Lucena et al., 2010; Serate et al., 2015).

Thus, this thesis firstly studied the use of the sugarcane bagasse as cell carrier for IBE production, to attack the fermentation productivity problem. Then, we applied the vacuum extraction technology to the sugarcane bagasse immobilized IBE fermentation to decrease butanol toxicity and enable more complete sugar conversion. A 3D printed cage-like polymeric structure was designed to trap the sugarcane bagasse inside the bioreactor and improve mass transfer efficiency between the biofilms adhered to the sugarcane bagasse and the fermentation broth during vacuum extraction. Additionally, we studied the use of second-generation feedstock, such as sugarcane bagasse cellulose and hemicellulose hydrolysates containing inhibitor compounds for IBE production.

1.2. Objectives

The main objective of this thesis was the development of an innovative fermentation technology for isopropanol, butanol and ethanol production. The fermentation system combined cell immobilization technology, where the sugarcane bagasse trapped in a 3D-printed cage-like polymeric structure was used as cell carrier, and in-situ product recovery by vacuum extraction. In parallel, sugarcane bagasse second-generation feedstock, i.e. cellulose and hemicellulose hydrolysates containing fermentation inhibitors, were used as substrate for IBE fermentation.

To accomplish this task, we determined the following specific objectives:

1. Evaluate the sugarcane bagasse as cell carrier for IBE production by *Clostridium beijerinckii* DSM 6423, and the long-term stability of immobilized cells in repeated-batch fermentation.
2. Evaluate different geometries for the 3D printed cage-like polymeric structure used to trap the sugarcane bagasse concerning mass transfer between the bagasse and the fermentation broth.
3. Evaluate the fermentation performance of the innovative technology composed by the immobilization system developed in the previous section coupled with in-situ product recovery by vacuum extraction.
4. Evaluate sugarcane bagasse second-generation feedstock as substrate to produce IBE; a cellulose hydrolysate containing lactic acid from microbial contamination and a hemicellulose hydrolysate from acid hydrolysis were used.

1.3. Document organization and main contributions

This document was organized in five chapters: (1) Introduction, (2) Publications and Future Publication, (3) Discussion, (4) Conclusions, and (5) References.

In chapter 1 we provide the context of the research conducted during this PhD, the

main objectives we planned to achieve, the organization of this document, and the list of publications in international journals and conferences.

Chapter 2 comprises our findings already published in international journals. In section 2.1 we conducted a review on IBE production, which was published by *Bioresource Technology* under the title of *Acetone-free biobutanol production: Past and recent advances in the Isopropanol-Butanol-Ethanol (IBE) fermentation*. Section 2.2 contains our findings about the use of sugarcane bagasse as cell holding material for IBE production. This study was published as *Isopropanol-butanol-ethanol (IBE) production in repeated-batch cultivation of Clostridium beijerinckii DSM 6423 immobilized on sugarcane bagasse* by the journal *Fuel*. Results concerning the use of sugarcane bagasse cellulose and hemicellulose hydrolysates as substrate for IBE production are presented in section 2.3. The paper related to this section was published by *Bioresource Technology* under the title of *Sugarcane bagasse hydrolysates as feedstock to produce the isopropanol-butanol-ethanol fuel mixture: Effect of lactic acid derived from microbial contamination on Clostridium beijerinckii DSM 6423*.

In chapter 3 we present the results of our future publication concerning the development of the innovative fermentation technology for IBE production using sugarcane bagasse trapped inside the 3D cage-like structure as cell holding material for *Clostridium beijerinckii* DSM 6423 in a bioreactor equipped with the vacuum extraction. The paper related to this section is still under preparation. In this chapter, we would like to offer a special acknowledgement to FAPESP (grant number 2016/23042-9) for providing financial resources for the participation in the 12th European Congress of Chemical Engineering occured in Florence, Italy (2019). The discussions about the anaerobic bioreactor setup suitable for *Clostridium beijerinckii* DSM 6423 specifications happened in this occasion were mandatory for concluding this part of the

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In chapter 4 we summarize the most important results and discussions. Chapter 5 comprises the conclusions of our findings and suggestions for future works, and in chapter 5 we list the references used in chapter 1.

JOURNAL PAPERS:

Paper 1: VIEIRA, C.F.S., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P., 2019. Acetone-free biobutanol production: Past and recent advances in the Isopropanol-Butanol-Ethanol (IBE) fermentation. Bioresour. Technol. 287, 121425.

Paper 2: VIEIRA, C.F.S., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P., 2020. Isopropanol-butanol-ethanol (IBE) production in repeated-batch cultivation of *Clostridium beijerinckii* DSM 6423 immobilized on sugarcane bagasse. Fuel, 263, 116708.

Paper 3: VIEIRA, C.F.S., CODOGNO, M.C., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P. Sugarcane bagasse hydrolysates as feedstock to produce the isopropanol-butanol-ethanol fuel mixture: Effect of lactic acid derived from microbial contamination on *Clostridium beijerinckii* DSM 6423. Bioresour. Technol. 319, 124140.

Paper 4*:* VIEIRA, C.F.S., DUZI, A.S., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P. Using 3D-printing to build the immobilization system of repeated-batch fermentation integrated with vacuum extraction for isopropanol-butanol-ethanol production (under preparation).

CONFERENCE PAPERS:

VIEIRA, C.F.S., CODOGNO, M.C., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P. Efeito do pré-tratamento alcalino no bagaço de cana como agente de imobilização celular para a produção de isopropanol, butanol e etanol. In: Congresso Brasileiro de Engenharia Química, São Paulo, 2018.

VIEIRA, C.F.S., CODOGNO, M.C., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P. Alkaline peroxide pretreated sugarcane bagasse as cell immobilization carrier for isopropanol-butanol-ethanol production. In: XXII European Congress of Chemical Engineering & V European Congress of Applied Biotechnology, Florence, 2019.

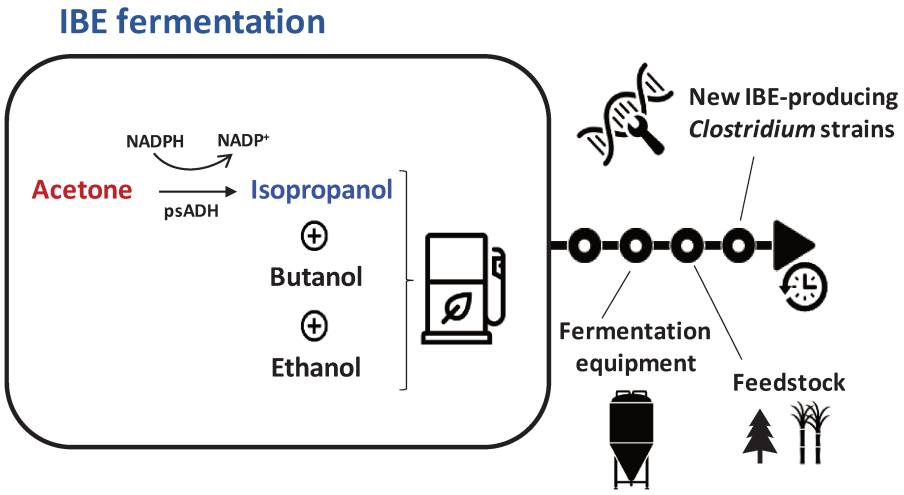
2. PUBLICATIONS

2.1. Acetone-free biobutanol production: Past and recent advances in the Isopropanol-Butanol-Ethanol (IBE) fermentation

This section comprises a literature review on past and recent advances in microorganisms, feedstocks, and fermentation equipment for IBE production.

Source:
VIEIRA, C.F.S., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P., 2019a. Acetone-free biobutanol production: Past and recent advances in the Isopropanol-Butanol-Ethanol (IBE) fermentation. Bioresour. Technol. 287, 121425.

Graphical Abstract



Abstract

Production of butanol for fuel via the conventional Acetone-Butanol-Ethanol fermentation has been considered economically risky because of a potential oversupply of acetone. Alternatively, acetone is converted into isopropanol by specific solventogenic *Clostridium* species in the Isopropanol-Butanol-Ethanol (IBE) fermentation. This route, although less efficient, has been gaining attention because IBE mixtures are a potential fuel. The present work is dedicated to reviewing past and recent advances in microorganisms, feedstock, and fermentation equipment for IBE production. In our analysis we demonstrate the importance of novel engineered IBE-producing *Clostridium* strains and cell retention systems to decrease the staggering number of fermentation tanks required by IBE plants equipped with conventional technology. We also summarize the recent progress on recovery techniques integrated with fermentation, especially gas stripping. In addition, we assessed ongoing pilot-plant efforts that have been enabling IBE production from woody feedstock.

Keywords: butanol, IBE fermentation, metabolic engineering, feedstock, advanced fermentation.

2.1.1. Introduction

The shortage of acetone during the World War I prompted the installation of the first Acetone-Butanol-Ethanol (ABE) plants (Jones and Woods, 1986); however, nowadays acetone creates uncertainty for investments in the ABE industry. This is because butanol has been considered an advanced biofuel and its production in a scale of billions of liters may result in oversupply of acetone. If butanol production reaches, for example, the size of the sugarcane ethanol market in Brazil (~30 billion liters per year), 12 million tons of acetone would also be produced annually (the ratio of butanol to acetone is roughly two in the ABE fermentation). This amount is approximately twice the global demand for acetone. An aggravating factor is that most of the oil-based acetone is obtained as a coproduct of phenol production, and acetone pricing, thus, depends on market conditions for phenol (Gröne et al., 2019). Furthermore, acetone does not qualify for government incentives to biofuels. Thus, unfavorable acetone prices are expected in case hundreds of ABE plants are installed to fulfill biofuel mandates.

There are, however, different solutions to the acetone issue (with pros and cons). For instance, ABE can be catalytically converted into fuel precursors and then hydrogenated, at additional cost, to fuel alkanes (Xie et al., 2019). Two other alternatives depend on metabolic engineering and seek ways (i) to decrease or eliminate acetone production in favor of better yields of butanol in the ABE fermentation (Zheng et al., 2015), and (ii) to develop acetone-free butanol production by *Saccharomyces cerevisiae* (Swidah et al., 2018; Zhao et al., 2018). Nonetheless, despite the intrinsic challenges of developing such microorganisms (Liu et al. 2018a), there is a risk they may not perform as expected under semi-sterile conditions found in commodity bioprocesses (Mariano, 2015). Acetone production can also be avoided by using *C. pasteurianum*, a non-acetone producing species that converts glycerol into butanol and

1,3-propanediol (PDO) (Biebl, 2001). However, the risk of oversupply may persist with PDO since its global demand is expected to be limited to 0.25 million ton in 2020 (Biddy et al., 2016).

Other two ways to mitigate the risk of a glut of acetone are based on its conversion into isopropanol. The chemical conversion has been conducted in times of surplus of acetone and when propylene, the primary feedstock, rose in price. But economic feasibility depends on access to low-cost hydrogen (Victory, 2010). The biological conversion, instead, can be achieved by naturally-occurring solventogenic strains able to reduce acetone to isopropanol in the Isopropanol-Butanol-Ethanol (IBE) fermentation. This alternative is appealing because IBE mixtures could be commercialized as an automotive fuel. Acetone, in contrast, is corrosive to rubber engine parts and has poor fuel properties (Li et al., 2019). Nonetheless, natural IBE producers are more sensitive to product inhibition (caused mainly by butanol) and, consequently, less efficient than ABE producers (Survase et al., 2013; Youn et al., 2016; Zhang et al., 2018).

For that reason, the acetone issue has been mostly addressed by metabolic engineering to decrease acetone production (in favor of butanol) by ABE producers (Liu et al. 2018a). The IBE fermentation has received considerably less attention and, in fact, the frequency of the term “ABE fermentation” in scientific articles over the last four decades (1982−2019) is nearly 20 times greater than that of “IBE fermentation” and “IB fermentation” (1,265 against 74 document results; source: Scopus Elsevier, 23 Jan. 2019). While the 1970s energy crisis may have triggered the research on both fermentation routes, low oil prices in the mid-eighties and the resulting scarcity of funding for renewable energy may explain why research on IBE fermentation virtually stopped for almost 20 years between 1990 and 2010. Meanwhile, research on ABE

fermentation was not abandoned and has been experiencing an exponential growth since the mid-2000s. At that time the oil price picked up again, and the United States created the Renewable Fuel Standard program, which secures market for advanced biofuels such as butanol. Such body of knowledge on ABE fermentation (reviewed by, for example, Jiang et al., 2015; Patakova et al., 2018; Jiménez-Bonilla and Wang, 2018; Xin et al., 2018; Kolesinska et al., 2019) has served as the basis for the renewed and growing interest in the IBE fermentation since the late 2000s. Most of the current research on IBE fermentation concentrates on (i) converting ABE producers into IBE producers through metabolic engineering, and (ii) the development of efficient bioreactors with cell immobilization and integrated product recovery. Advances have also been made in the use of agricultural and forest biomass feedstocks and industrial wastes for IBE production. In this review we examine the advances and document the technology development timeline for IBE fermentation.

2.1.2. Overview of the advances in IBE fermentation

2.1.2.1. Past advances

In the 1980s, metabolic engineering tools for butanol-producing *Clostridium* species were in their infancy and for this reason the first attempts to improve the performance of IBE producers concentrated on the design of advanced bioreactors (Figure 2.1.1). These studies were mostly led by researchers from the Delft University of Technology (aka TU Delft) in The Netherlands. They developed different immobilized cell bioreactors, namely continuous column, fluidized, and gas lift loop reactors. The design of the bioreactors was probably inspired by the research on ethanol and ABE fermentations conducted at that time, which was also seeking to improve fermentation performance by immobilization of cells on calcium alginate (e.g., Shiotani and Yamané, 1981; Förberg et al., 1983; Frick and Schügerl, 1986). At TU Delft they

also studied the integrated recovery of IBE by two techniques: adsorption and pervaporation. Interestingly, their work on pervaporation was the only study reported so far on process control of IBE fermentation (van der Heiiden et al., 1989). Pervaporation was also the choice of researchers from the University of Tsukuba (Japan) in the early 1990s just before the 20-year hiatus of publications on IBE fermentation.

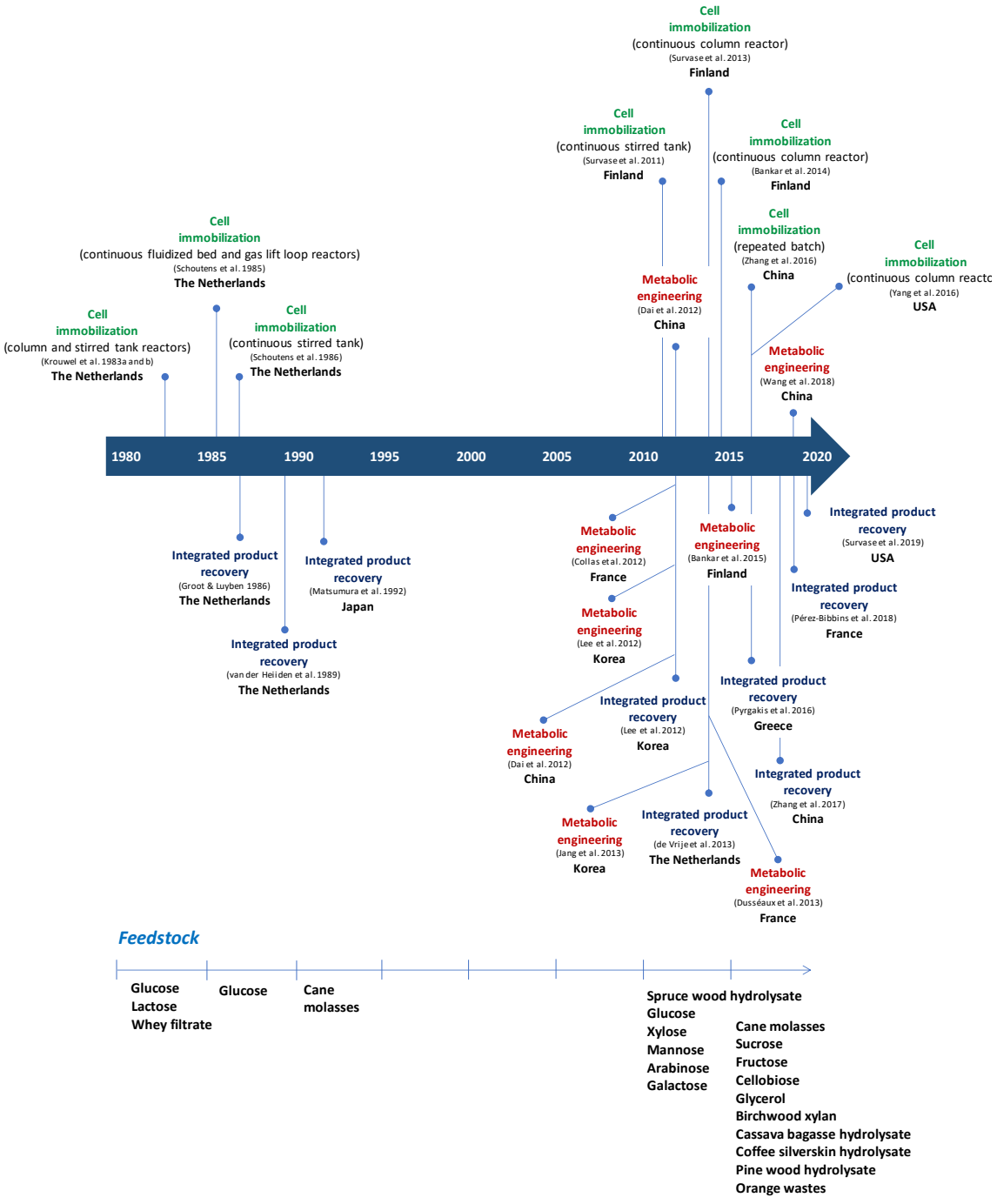


Figure 2.1.1 – Technology development timeline for IBE fermentation.

2.1.2.2. Recent advances

Research on IBE fermentation resumes in the early 2010s, and since then new strategies of cell immobilization and integrated product recovery have been developed. Furthermore, a new research frontier has emerged: metabolic engineering to convert ABE-producing *Clostridium* species into IBE producers (Figure 2.1.1). Regarding fermentation equipment, researchers from the Aalto University in Finland have achieved important gains in IBE productivity by developing cell immobilization systems in continuous column reactors using renewable materials as cell carrier (e.g., wood pulp, sugarcane bagasse, coconut fibers). As for the integrated product recovery, research conducted in different countries (Korea, China, France, Greece, The Netherlands, USA) has focused on gas stripping and liquid-liquid extraction to improve IBE titer. Research on metabolic engineering has been mostly led by universities in South Korea and China. This is not surprising because China imports most of its butanol and consumes one third of the global production (Jiang et al., 2015); moreover, China is probably the only country that currently has ABE plants. Metabolic engineered IBE producers and integrated liquid-liquid extraction are also the choices of the US-based cleantech process development company American Process Inc. (API), where pioneering pilot efforts have been led by former Aalto University researcher Dr. Shrikant Survase (Survase et al., 2019).

The IBE fermentation is also advancing in terms of feedstock. While the first round of technology development in the 1980s was focused on fermentation equipment (except for attempts to use whey filtrate and sugarcane molasses), today's research on IBE fermentation is also giving attention to lignocellulosic feedstock and industrial wastes. Significant progress has been made with respect to woody biomass, especially softwoods (spruce and southern pine). These works initiated at the time Professor

Adriaan van Heiningen of University of Maine (USA) was serving as visiting research professor at Aalto University during the early 2010s. He and his collaborators were developing a SO_2-ethanol-water fractionation process that is particularly suitable to produce sugars from softwoods. This process is currently commercialized by API under the brand name AVAP® and has been serving their IBE pilot plant (Survase et al., 2019).

In the next sessions we dive into analyzing the recent advances.

2.1.3. Engineered IBE-producing *Clostridium* strains

2.1.3.1. Improved isopropanol producers

The development of new IBE-producing *Clostridium* strains has been prompted by the poor performance of wild-type strains. In batch culture of natural IBE producers [which are mainly *Clostridium beijerinckii* strains (Chen and Hiu, 1986)], butanol concentration rarely exceeds 6 g/L and production is slow (< 0.2 g IBE/L·h) (Table 2.1.1). Hitherto the best results were provided by *C. beijerinckii* BGS1 (isolated from soil from grass land), which produced 10.2 g/L butanol and 3.4 g/L isopropanol (Zhang et al., 2018). In contrast, 12−13 g/L butanol is usually delivered by wild-type ABE producers (Mariano et al., 2015).

Table 2.1.1 – Performance of IBE batch fermentation according to carbon source and *Clostridium* strain.

Carbon source	g/L	Strain	IBE yield (g/g sugar)	IBE productivity (g/L·h)	B (g/L)	I (g/L)	Reference
Glucose	20	*C. beijerinckii* VPI 2968	-[a]	-	3.3	0.6	George et al. (1983)
	20	*C. beijerinckii* VPI 2982	-	-	3.0	0.1	George et al. (1983)
	20	*C. butylicum* NRRL B-593	-	-	4.6	0.5	George et al. (1983)
	20	*C. aurantibutyricum* ATCC 17777	-	-	3.4	0.3	George et al. (1983)
	20	*C. aurantibutyricum* NCIMB 10659	-	-	3.1	0.6	George et al. (1983)
	20	*C. beijerinckii* VPI 2432	-	-	6.0	1.6	Chen and Hiu (1986)
	20	*C. beijerinckii* VPI 4771	-	-	5.6	1.2	Chen and Hiu (1986)
	20	*C. beijerinckii* VPI 13114	-	-	5.3	1.4	Chen and Hiu (1986)
	20	*C. beijerinckii* VPI 13105	-	-	5.3	0.8	Chen and Hiu (1986)
	120	*C. beijerinckii* LMD 27.6	-	-	5.4	2.7	Groot and Luyben (1986)
	60	*C. beijerinckii* DSM 6423	0.30	-	3.7	2.2	Survase et al. (2011)
	20	*Clostridium* sp. A1424	0.32	0.25	4.5	2.5	Youn et al. (2016)
	30	*C. beijerinckii optinoii*	0.39	0.10	6.2	3.2	Yang et al. (2016)
	60	*C. beijerinckii* BGS1	-	-	10.2	3.4	Zhang et al. (2018)
Glucose and xylose	40/20	*C. beijerinckii* NRRL B593	0.32	0.16	6.9	3.2	Vrije et al. (2013)
Mixed sugars	48[b]	*C. beijerinckii* DSM 6423	0.25	0.08	2.8	1.2	Survase et al. (2013)
Mannose	20	*Clostridium* sp. A1424	0.34	0.22	4.4	1.9	Youn et al. (2016)
Fructose	20	*Clostridium* sp. A1424	0.31	0.21	4.4	1.6	Youn et al. (2016)
Cellobiose	20	*Clostridium* sp. A1424	0.37	0.25	4.5	2.6	Youn et al. (2016)
Sucrose	20	*Clostridium* sp. A1424	0.37	0.24	5.0	1.7	Youn et al. (2016)
	60	*C. beijerinckii* BGS1	-	-	9.8	2.5	Zhang et al. (2018)
Cane molasses	30	*C. beijerinckii optinoii*	0.39	0.14	7.6	4.6	Moon et al. (2015)
Lactose	42	*C. beijerinckii* LMD 27.6	0.13	0.02	1.5	0.2	Schoutens et al. (1984)
Whey ultrafiltrate (80% lactose)	50	*C. beijerinckii* LMD 27.6	0.24	0.04	5.0	0.9	Schoutens et al. (1984)
Cassava bagasse hydrolysate	30[c]	*C. beijerinckii* ATCC 6014	-	-	8.2	4.3	Zhang et al. (2016)
Coffee silverskin hydrolysate	21[d]	*C. beijerinckii* DSM 6423	0.47	0.13	4.4	2.2	Procentese et al. (2018)
Birchwood xylan	60	*C. beijerinckii* NJP7	-	0.05	2.1	0.5	Xin et al. (2017)

[a] not available; [b] (in g/L) glucose 8.5, mannose 22.0, arabinose 2.3, galactose 4.5, and xylose 10.5; [c] (in g/L) glucose 13.5, mannose 2.2, arabinose 1.1, fructose 2.8, and xylose 10.2; [d] hydrolysate contained only glucose.

Since solventogenic *Clostridium* species are more tolerant to isopropanol than butanol, research has been conducted to transform IBE-producing *Clostridium* strains in hyper-isopropanol strains. Gérando et al. (2016) used random mutagenesis and genome shuffling to increase the tolerance of *C. beijerinckii* DSM 6423 to isopropanol (from 35 to 50 g/L). However, the enhanced tolerance was not accompanied by improved fermentation performance. Despite that, the same research group recently described the complete genome sequence of *C. beijerinckii* DSM 6423 and performed the first transcriptome analysis of that strain (Gérando et al., 2018). They expect *C. beijerinckii* DSM 6423 will become a microbial cell factory for isopropanol production.

2.1.3.2. Transformation of ABE- into IBE-producing strains

The development of hyper-IBE producers has been more effective when transforming hyper ABE-producing strains into IBE producers. The transformation consists in inserting in ABE producers the gene (sadh) that encodes the enzyme (primary-secondary alcohol dehydrogenase, psADH) responsible for reducing acetone to isopropanol in IBE producers. This strategy has so far only been applied to *C. acetobutylicum*. Various strains of this species were used in ABE plants, and they have been the preferred model organism in metabolic engineering studies of solventogenic clostridia since the early 2000s (Nölling et al., 2001; Papoutsakis, 2008; Lütke-Eversloh and Hubert Bahl, 2011; Cho et al., 2015).

Thus, highly butanol-tolerant *C. acetobutylicum* strains, previously developed with focus on ABE production, have been now used as platform for engineering IBE-producing *Clostridium* strains (Table 2.1.2). For instance, solvent production by *C. acetobutylicum* Rh8 (mutant strain of *C. acetobutylicum* DSM 1731) changed from 20.5 g/L ABE (g/L, 3.5 A, 15.5 B, 1.5 E) to 23.9 g/L A(I)BE (g/L, <0.02 A, 7.6 I, 15.0 B, 1.3 E) after an one-step engineering approach that expressed the gene sadh in that strain

(Dai et al., 2012). In another successful example of transformation, solvent production by *C. acetobutylicum* BKM19 changed from 32.5 g/L ABE (g/L, 4.4 A, 17.6 B, 10.5 E) to 28.5 g/L IBE (g/L, 3.5 I, 15.4 B, 9.6 E) in a 200-L bioreactor (Jang et al., 2013a and b).

However, engineered *C. acetobutylicum* strains may not always respond as expected during scale-up. For example, in laboratory-scale experiments (batch and chemostat cultivations), *C. acetobutylicum* DSM 792-ADH was able to convert approximately 50% of acetone to isopropanol (Bankar et al., 2015). But in pilot tests recently conducted at American Process Inc., conversion dropped to 14% after a few hours in continuous operation mode. The incomplete conversion was attributed to a possible redox imbalance since the reaction that converts acetone into isopropanol needs NADPH (Survase et al., 2019). Therefore, it is not surprising that potential failures of genetically modified microorganisms have been considered an important risk factor during assessment of emerging fermentation technologies (Mariano, 2015).

Table 2.1.2 - Metabolic engineering for IBE production.

Gene	Encoding enzyme	Mode of genetic modification	Host	Expression result	Reference
sadh, adc, ctfA, ctfB	Primary-secondary alcohol dehydrogenase, acetoacetate decarboxylase, acetoacetyl-CoA: acetate/butyrate: CoA transferase subunits A and B	Insertion	*C. acetobutylicum* ATCC 824 20.2 g/L A(I)BE (g/L, 7.6 A, 0.1 I, 11.6 B, 0.9 E) 90 g/L initial glucose concentration A(I)BE yield: 0.31 g/g; A(I)BE productivity: 0.38 g/L·h	*C. acetobutylicum* ATCC 824(pFC007) 24.4 g/L A(I)BE (g/L, 0.1 A, 8.8 I, 13.7 B, 1.5 E) 90 g/L initial glucose concentration A(I)BE yield: 0.35 g/g; A(I)BE productivity: 0.80 g/L·h	Collas et al. (2012)
sadh	Primary-secondary alcohol dehydrogenase	Insertion	*C. acetobutylicum* Rh8 (mutant strain of *C. acetobutylicum* DSM 1731) 20.5 g/L A(I)BE (g/L, 3.5 A, 0 I, 15.5 B, 1.5 E) 80 g/L initial glucose concentration B yield: 0.20 g/g; A(I)BE productivity: 0.25 g/L·h	*C. acetobutylicum* Rh8(psADH) 23.9 g/L A(I)BE (g/L, <0.02 A, 7.6 I, 15 B, 1.3 E) 80 g/L initial glucose concentration A(I)BE yield: 0.31 g/g; A(I)BE productivity: 0.48 g/L·h	Dai et al. (2012)
sadh	Primary-secondary alcohol dehydrogenase	Insertion	*C. acetobutylicum* PJC4BK 19 g/L A(I)BE (g/L, 3.7 A, 0 I, 12.9 B, 2.4 E) 80 g/L initial glucose concentration A(I)BE yield: 0.28; A(I)BE productivity: 0.63 g/L·h	*C. acetobutylicum* ATCC 824 PJC4BK(pIPA3-Cm2) [1] 20.4 g/L A(I)BE (g/L, 0 A, 4.4 I, 14.1 B, 1.9 E) 80 g/L initial glucose concentration A(I)BE yield: 0.30 g/g; A(I)BE productivity: 0.68 g/L·h	Lee et al. (2012)
sadh	Primary-secondary alcohol dehydrogenase	Insertion and overexpression	*C. acetobutylicum* ATCC 824 *Δcac15ΔuppΔbuk* 20.1 g/L A(I)BE (g/L, 3.9 A, 0 I, 14.7 B, 1.5 E) 80 g/L initial glucose concentration A(I)BE yield: 0.28 g/g; A(I)BE productivity: -	*C. acetobutylicum* ATCC 824 Δbuk pCLF952 21 g/L A(I)BE (g/L, 5.0 I, 14 B, 1.1 E) 80 g/L initial glucose concentration A(I)BE yield: 0.34 g/g; A(I)BE productivity: 0.80 g/L·h	Dusséaux et al. (2013)
sadh, hydG	Primary-secondary alcohol dehydrogenase, putative electron transfer protein	Insertion	*C. acetobutylicum* BKM19 (hyper ABE producer) 32.5 g/L A(I)BE (g/L, 4.4 A, 17.6 B, 10.5 E)	*C. acetobutylicum* BKM19 (pIPA100) 28.5 g/L A(I)BE (g/L, 3.5 I, 15.4 B, 9.6 E)	Jang et al. (2013a)

			85.2 g/L initial glucose concentration A(I)BE yield: 0.38 g/g; A(I)BE productivity: 0.61 g/L·h	76 g/L initial glucose concentration (200-L fermentor) A(I)BE yield: 0.37 g/g; A(I)BE productivity: 0.47 g/L·h	
sadh	Primary-secondary alcohol dehydrogenase	Insertion	*C. acetobutylicum* DSM 792 Performance not available	*C. acetobutylicum* DSM 792-ADH 15.0 g/L A(I)BE (g/L, 2.6 A, 2.5 I, 8.6 B, 1.3 E) 60 g/L initial glucose concentration A(I)BE yield: 0.34 g/g; A(I)BE productivity: 0.10 g/L·h	Bankar et al. (2015)
sadh	Primary-secondary alcohol dehydrogenase	Insertion	*C. acetobutylicum XY16* Performance not available	*C. acetobutylicum* XY6 (pSADH) 16.1 g/L A(I)BE (g/L, 5.0 I, 10 B, 1.1 E) 60 g/L initial glucose concentration A(I)BE yield: 0.31 g/g; A(I)BE productivity: 0.22 g/L·h	Wang et al. (2018)

[1] Velázquez-Sánchez et al. (2019) developed a kinetic model that describes the effect of pH of the culture medium on IBE production by *C. acetobutylicum* pIPA3-Cm2. This is the first model of this kind and opens the opportunity for studies on design, optimization, and control of IBE-producing systems.

2.1.4. Feedstock for IBE production

Inasmuch as the research on IBE fermentation is still mainly focused on delivering fermentation systems as efficient as those with ABE-producing strains, many of the recent studies on IBE fermentation have been using laboratory-grade glucose as carbon source. This approach takes advantage from the fact that glucose is generally more metabolizable than xylose, and it eliminates effects of lignocellulose-derived microbial inhibitory compounds. Such simplification, which is characteristic of initial research steps, has mainly been adopted in metabolic engineering studies for IBE production. Meanwhile, metabolic engineering has already enhanced the tolerance of ABE-producing *Clostridium* species to biomass-derived inhibitors such as phenolic compounds (Liu et al., 2018b). Nonetheless, as described below, important advances regarding feedstock for IBE production include the use of agricultural and forest biomass feedstocks and industrial wastes.

2.1.4.1. Agricultural and forest biomass feedstocks

As demonstrated by the extensive body of knowledge on feedstock for ABE production [a review can be found in Kolesinska et al. (2019)], IBE fermentation of complex carbon sources such as hydrolysates of lignocellulosic material is also challenging. In the early 2010s, before starting their work with wood hydrolysates, Aalto University researchers found that *C. beijerinckii* DSM 6423 was not able to exhaust a sugar mixture (48 g/L; glucose, mannose, galactose, arabinose, and xylose) that mimicked lignocellulose hydrolysate (Survase et al., 2013). Moreover, glucose was consumed preferentially and IBE productivity was as low as 0.08 g/L·h (in batch mode). Such challenge they also observed when fermenting spruce wood hydrolysate. In a continuous culture of *C. acetobutylicum* DSM792-ADH, the highest IBE concentration

(6 g/L) obtained from the hydrolysate was significantly lower than those from glucose (10.6 g/L) and mixed sugars (10.0 g/L) (Bankar et al., 2014) (Table 2.1.1).

More recently, the modified strain *C. acetobutylicum* DSM792-ADH has been used in the pilot tests at American Process Inc. to ferment pine wood hydrolysate (Survase et al., 2019). In their pilot plant, the cellulose (C6 sugars) and hemicellulose (C5 sugars) hydrolysates are fermented in different continuous fermentors to avoid the incomplete glucose utilization they observed during laboratory fermentation of mixed C5 and C6 hydrolysates. This strategy is accompanied by cell recycling and, besides delivering an IBE productivity as high as 10 g/L·h, it allowed to overcome the carbon catabolite repression mechanism commonly found in solventogenic clostridia. Because of this mechanism, rapidly metabolizable sugars (e.g., glucose) are consumed preferentially, and the presence of these sugars inhibits the expression of genes and enzymes responsible for the catabolism of non-preferred sugars such as xylose (Ren et al., 2016). Another advantage in fermenting the C5 and C6 sugars separately is that it allows for process flexibility. In our research group, we recently proposed an ethanol-butanol flexible process based on the different abilities of *Saccharomyces cerevisiae* and ABE-producing strains to metabolize C6 and C5 sugars (Pereira et al., 2018; Assumpção et al., 2018). We found that this concept is economically advantageous in relation to either ethanol- or ABE-dedicated plants because the cellulose hydrolysate can be used for either ethanol or ABE production according to market price variations. The hemicellulose hydrolysate, on the other hand, should be exclusively reserved for ABE (or IBE) production given that industrial *S. cerevisiae* strains cannot ferment C5 sugars.

Promising results were also obtained with birchwood xylan (Xin et al., 2017; Jiang et al., 2018) and agricultural residues [cassava bagasse (Zhang et al., 2016) and

coffee silverskin (Procentese et al., 2018)]. With respect to xylan, it was found that *C. beijerinckii* NJP7 wild-type strain secretes extracellular xylanases and, thus, can convert hemicellulose directly to butanol and isopropanol (Jiang et al., 2018). This strain was able to partially convert 60 g/L birchwood xylan into 0.5 g/L isopropanol, 2.1 g/L butanol, and 3.2 g/L ethanol in 120 h (Xin et al., 2017). These findings open the opportunity for IBE production from hemicellulose in consolidated bioprocessing, i.e. the conversion of hemicellulose into IBE in one step without adding hydrolyzing agents (inorganic acids, enzymes). As for the cassava bagasse, this feedstock is rich in starch, cellulose, and hemicellulose, and these polysaccharides were converted into glucose and xylose using only dilute sulfuric acid pretreatment, i.e. enzymatic hydrolysis was not needed (Zhang et al., 2016). The resulting cassava bagasse hydrolysate (30 g/L total sugars) was then efficiently converted by *C. beijerinckii* ATTC 6014 into butanol (8.2 g/L) and isopropanol (4.3 g/L) (Table 2.1.1). In the instance of coffee silverskin, this material, rich in lignin, is recalcitrant. As such, coffee silverskin was pretreated in an alkaline process (to remove lignin and hemicellulose) and enzymatic hydrolysis converted cellulose into glucose (Procentese et al., 2018). The resulting hydrolysate containing low glucose concentration (20 g/L) was partially converted (67%) by *C. beijerinckii* DSM 6423 into butanol (4.4 g/L) and isopropanol (2.2 g/L). We speculate that glucose was not exhausted because citrate buffer (100 mM) was used in the enzymatic hydrolysis. Although citrate buffer is commonly used in studies on lignocellulosic ethanol fermentation, it inhibits the growth of ABE-producing strains such as *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 (Liu et al., 2015).

In fact, gains in IBE production are expected if acetate buffer is used for enzymatic hydrolysis instead. In reference to ABE fermentation, the use of acetate

buffer in the hydrolysis of Eastern redcedar resulted in an increase of 3−4 g/L butanol in relation to the experiment without acetate buffer (Liu et al., 2015). With regards to IBE fermentation, the French research center IFP Energies Nouvelles patented a process for IBE production from C5 and C6 sugars based on the addition of acetate (US20170137848A1, "IBE Fermentation Method"). The inventors cultivated *C. beijerinckii* NRRL B593 (DSMZ 6423) in defined fermentation medium containing 60 g/L glucose and different concentrations of ammonium acetate (0; 3; and 6 g/L). In both the control and the experiment with ammonium acetate (6 g/L, without pH control) glucose conversion was approximately 60% after 50 h. However, due to consumption of acetate, IBE concentration increased from 8.9 (control) to 16.6 g/L. The patent claims that the pH of the aqueous solution containing acetate (i.e. acetic acid in the deprotonated form) must be in the range 5 to 8. They also described potential sources of acetic acid in plants that process lignocellulosic feedstock: effluent from pretreatment and hydrolysate obtained by enzymatic hydrolysis. Indeed, hemicellulose hydrolysates produced by several pretreatment technologies, including dilute sulfuric acid, contain acetic acid, which results from the hydrolysis of acetyl groups (Jönsson and Martín, 2016).

2.1.4.2. Industrial wastes

The following industrial wastes have been assessed for IBE production: whey filtrate (a by-product of cheese production containing mainly lactose), orange wastes, sugarcane molasses, and glycerol. With regards to whey filtrate, a study in the early 1980s found that IBE batch production by *C. beijerinckii* LMD 27.6 using whey filtrate was three times slower than that using glucose (Schoutens et al., 1984) (Table 2.1.1). To produce IBE from orange wastes, a consolidated process using the cellulose-degrading bacterium *C. cellulovorans* and *C. beijerinckii* has been recently proposed (Tomita et

al., 2019). However, the resulting reducing sugar concentration (1.68 g/L) and butanol titer (0.28 g/L) are still far from being economically viable.

The potential of using sugarcane molasses for IBE production was firstly demonstrated by Matsumura et al. (1992); however, the molasses, which is rich in nutrients, was further supplemented with $(NH_4)_2SO_4$, $CaCO_3$, $CaC1_2.2H_2O$, peptone, and yeast extract. In contrast, Moon et al. (2015) demonstrated more recently that *C. beijerinckii optinoii* can produce IBE from sugarcane molasses without supplementary nutrients. *C. beijerinckii optinoii* exhausted 30 g/L sugars in 72 h, and IBE yield (0.39 g/g) was close to the yield obtained in the fermentation with supplementary nutrients (0.41 g/g). Treatment of the molasses with invertase (to convert sucrose into glucose and fructose) was needed to improve sugar conversion from 95% to 100%. Molasses, thus, may also become a convenient source of nutrients for future bagasse-based IBE plants annexed to sugarcane mills.

As for glycerol, the addition of this compound to sugar-based IBE fermentations can increase the conversion of acetone into isopropanol in cases in which IBE-producing strains can also metabolize glycerol. The reduction of acetone to isopropanol by primary-secondary alcohol dehydrogenase is NADPH dependent (Ismaiel et al., 1993), and the high reduction state of glycerol results in additional reducing equivalents (NADPH) during glycerol metabolism as compared to less reduced substrates such as glucose (Pyne et al., 2014). As such, *Clostridium* sp. A1424 (which is capable of consuming glycerol and produces small amounts of acetone, 0.5–1.5 g/L, when glucose is the sole carbon source) was able to achieve acetone-free production of IBE when glycerol was added to the fermentation medium (Youn et al., 2016).

2.1.5. Fermentation equipment

While feedstock price, feedstock processing, and fermentation yield (the last being determined mainly by characteristics of microorganisms and substrates) are major cost components of bio-based butanol (Mariano et al., 2013), fermentation equipment and operation mode can improve butanol titer and productivity. These less impactful parameters (that is, unless learning curve effects do not lower the cost of lignocellulosic sugar) cannot be neglected, though. They affect energy efficiency of downstream product separation, water and wastewater footprint, and capital investment (number of fermentors) (Mariano and Maciel Filho, 2012). The relationship is simple: energy and environmental efficiencies are improved as more concentrated in sugar the fermentation broth is; this operating condition results in a more concentrated product stream and increases fermentor productivity. To make it possible, diverse technologies have been developed to recover butanol during fermentation since sugar load in clostridial fermentation is dictated by the toxicity of butanol. They include adsorption, pervaporation, liquid–liquid extraction, perstraction, gas stripping, vacuum fermentation, flash fermentation, and reactive extraction (Jiménez-Bonilla and Wang, 2018). But only few of these technologies have been applied to IBE fermentation. Fermentor productivity also, or mainly, benefits from continuous operation and cell retention systems. Nevertheless, advances in bioreactors have been likewise focused on ABE fermentation (Mariano et al., 2015). Despite that, important gains for IBE fermentation have been achieved with fermentation equipment, as described as follows.

2.1.5.1. Bioreactors with cell retention

Cell retention systems have been developed since the early 1980s to improve the productivity of IBE-producing *Clostridium* strains (Table 2.1.3). Early studies at TU Delft used the immobilization technique of entrapment in calcium-alginate in different

continuous reactors (stirred tank, column, fluidized bed, and gas lift loop reactors). Notably, in a three-part article were presented the modeling, hydrodynamics, and laboratory- and pilot-scale experiments of fluidized bed and gas lift loop reactors (Schoutens et al., 1986a, b, and c). In those studies, which we consider the most exhaustive studies on bioreactor design for butanol production, IBE productivity achieved remarkable values between 1 to 3 g/L·h. Nonetheless, these configurations have not been revisited since then.

Current research efforts have focused on tank and column configurations, which have advantages such as simplicity and low cost of operation (Mariano et al., 2015). Other advantage explored nowadays is the immobilization technique of adsorption. This is a simple technique in which cells adsorb onto surfaces of solid materials (Figure 2.1.2). This strategy was extensively studied at Aalto University in the early 2010s, adding the advantage of choosing low-cost renewable materials such as wood pulp and sugarcane bagasse (Survase et al., 2011 and 2013; Bankar et al., 2014). For example, in a continuous column reactor packed with wood pulp operated for 25 days, conversion of mixed sugars by *C. beijerinckii* DSM 6423 achieved IBE productivity of 5.58 g/L·h (dilution rate of 1.5 h^{-1}). But butanol concentration was as low as 2.3 g/L and substrate conversion only 23% (Survase et al., 2013).

Table 2.1.3 – Performance of IBE fermentation systems with immobilized *Clostridium* cells.

Cell carrier	Carbon source	g/L	Operation mode	Strain	IBE yield (g/g sugar)	IBE productivity (g/L·h)	B (g/L)	I (g/L)	Reference
Ca-alginate	Glucose	30	Continuous (stirred tank)	*C. beijerinckii* LMD 27.6	0.34	3.10	-[a]	-	Krouwel et al. (1983a)
	Glucose	50	Continuous (fluidized bed reactor)	*Clostridium spp.* DSM 2152	-	1.0-3.0	5.8	3.3	Schoutens et al. (1986c)
	Glucose	50	Continuous (gas lift loop reactor)	*Clostridium spp.* DSM 2152	-	1.0-3.0	5.4	3.0	Schoutens et al. (1986c)
	Glucose	20	Continuous (column reactor)	*C. beijerinckii* LMD 27.6	0.34	0.58	5.0	3.0	Krouwel et al. (1983b)
	Whey ultrafiltrate (80% lactose)	25	Continuous (stirred tank)	*C. beijerinckii* LMD 27.6	-	0.5-1.0	2.0	0.5	Schoutens et al. (1985)
Ceramic Raschig rings	Glucose	30	Continuous (column reactor)	*C. beijerinckii optinoii*	0.51	1.03	7.6	4.5	Yang et al. (2016)
Cotton[b]	Cassava bagasse hydrolysate[c]	52	Batch (column reactor)	*C. beijerinckii* ATCC 6014	0.43	0.31	12.3	6.7	Zhang et al. (2016)
	Cassava bagasse hydrolysate[c]	52	Repeated batch (column reactor)	*C. beijerinckii* ATCC 6014	0.45	0.36	12.3	6.9	Zhang et al. (2016)
	Cassava bagasse hydrolysate[c]	61	Repeated batch (two column reactors in series)	*C. tyrobutyricum* ATCC 25755 (upstream column) and *C. beijerinckii* ATCC 6014 (downstream column)	0.50	0.43	13.1	7.6	Zhang et al. (2016)
Wood pulp	SO_2-ethanol-water spent liquor from spruce wood chips[d]	60	Continuous (column reactor)	*C. acetobutylicum*-DSM792-ADH	0.14	1.67	2.0	0.5	Bankar et al. (2014)
	Glucose	60	Continuous (stirred tank)	*C. beijerinckii* DSM 6423	0.29	5.52	6.1	4.8	Survase et al. (2011)
	Mixed sugars[e]	48	Batch	*C. beijerinckii* DSM 6423	-	-	10.1	6.0	Survase et al. (2013)
	Mixed sugars[e]	48	Continuous (column reactor)	*C. beijerinckii* DSM 6423	0.35	5.58	2.3	1.5	Survase et al. (2013)
Coconut fibers	Mixed sugars[e]	48	Batch	*C. beijerinckii* DSM 6423	-	-	8.5	5.7	Survase et al. (2013)
Wood chips	Mixed sugars[e]	48	Batch	*C. beijerinckii* DSM 6423	-	-	9.4	5.0	Survase et al. (2013)

Sugarcane bagasse	Mixed sugars[e]	48	Batch	*C. beijerinckii* DSM 6423	-	-	9.4	5.2	Survase et al. (2013)
Loofah sponge	Mixed sugars[e]	48	Batch	*C. beijerinckii* DSM 6423	-	-	10.1	5.7	Survase et al. (2013)

[a] not available;
[b] batch reactor connected to an external glass column packed with spirally wound cotton towel;
[c] glucose, mannose, arabinose, fructose, and xylose;
[d] hydrolysate was detoxified, diluted, and supplemented with sugars;
[e] (in g/L) glucose 8.5, mannose 22.0, arabinose 2.3, galactose 4.5, and xylose 10.5.

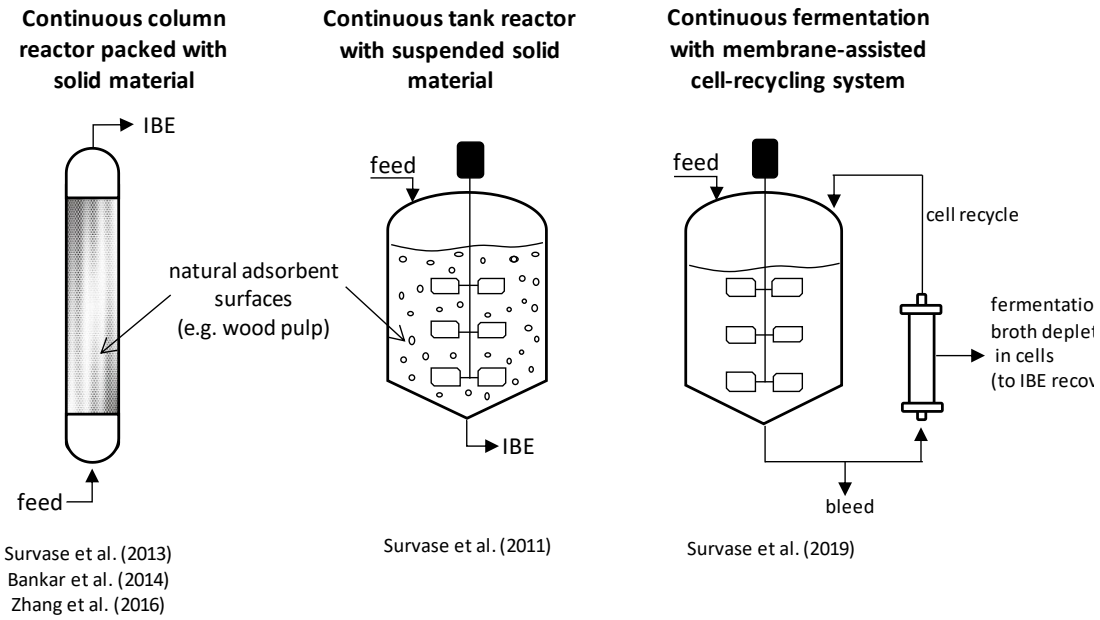


Figure 2.1.2 – Representative bioreactors with cell retention used for IBE production.

Indeed, improved productivity offered by continuous systems generally comes at the cost of dilute product streams. To circumvent this problem, research conducted after the studies from Aalto University has penalized productivity by either decreasing dilution rate or changing the operation mode altogether. For instance, in a continuous column reactor packed with ceramic Raschig rings and operated with dilution rate of 0.085 h^{-1} for 120 days, IBE productivity by *C. beijerinckii optinoii* was 1.0 g/L·h, and butanol concentration was 7.6 g/L (Yang et al., 2016). This concentration is rarely achieved in batch cultures of natural IBE producers (Table 2.1.1). In another example, concentrated cassava bagasse hydrolysate (52 g/L sugars) was processed in a column reactor packed with spirally wound cotton towel operated batch-wise. In five repeated batches IBE production by *C. beijerinckii* ATCC 6014 was relatively stable. Butanol concentration remarkably achieved 12 g/L; however, IBE productivity was 0.36 g/L·h (Zhang et al., 2016). That study also proposed a two-stage fermentation system with two column reactors packed with cotton. The upstream reactor was conceived to produce acetic acid by *C. tyrobutyricum*, and in the downstream reactor cells of *C.*

beijerinckii ATCC 6014 were responsible to convert the acid and remaining sugars into IBE. In repeated batches of the co-culture fermentation system, both butanol concentration (13.1 g/L) and IBE productivity (0.43 g/L·h) improved in relation to the single-stage system. Nonetheless, complete conversion of sugars remains a challenge even at lower productivity rates.

The observed penalties in IBE productivity of cell retention systems (in favor of butanol titer) are certainly advantageous, nevertheless. One important advantage is the saving in energy consumption. In the ABE fermentation, as a straightforward reference, the energy needed to distillate ABE increases exponentially if butanol concentration in the fermentation broth is lower than 10 g/L butanol (Mariano and Maciel Filho, 2012). Another advantage is that the number of fermentation tanks to be installed in an IBE plant may still be economically reasonable. To illustrate the last point, we estimated the number of fermentation tanks required by an IBE plant to produce 100 kton IBE per year (350 days) as a function of IBE productivity (Figure 2.1.3). Any operation downtime was ignored, and the size of each tank is 3785 m^3. This same size was considered in the design of a corn stover-to-ethanol plant by the National Renewable Energy Laboratory (NREL), in which the production of 182 kton/y ethanol required 12 tanks with ethanol productivity of 1.5 g/L·h (Humbird et al., 2011). We also used that reference to estimate the installation cost of each tank (3 MMUS$). Accordingly, in the range of IBE productivity achieved in batch cultures of natural IBE producers (< 0.20 g/L·h), the number of fermentors varies exponentially from 16 (if IBE productivity is 0.20 g/L·h) to staggering 63 tanks if productivity is 0.05 g/L·h [should IBE be produced from birchwood xylan in a consolidated bioprocess, Xin et al. (2017)]. This analysis suggests, for example, that a consolidated bioprocess to produce IBE directly from

hemicellulose may still not be economically feasible, especially when considering that hydrolysis of xylan during pretreatment of biomass is generally not difficult.

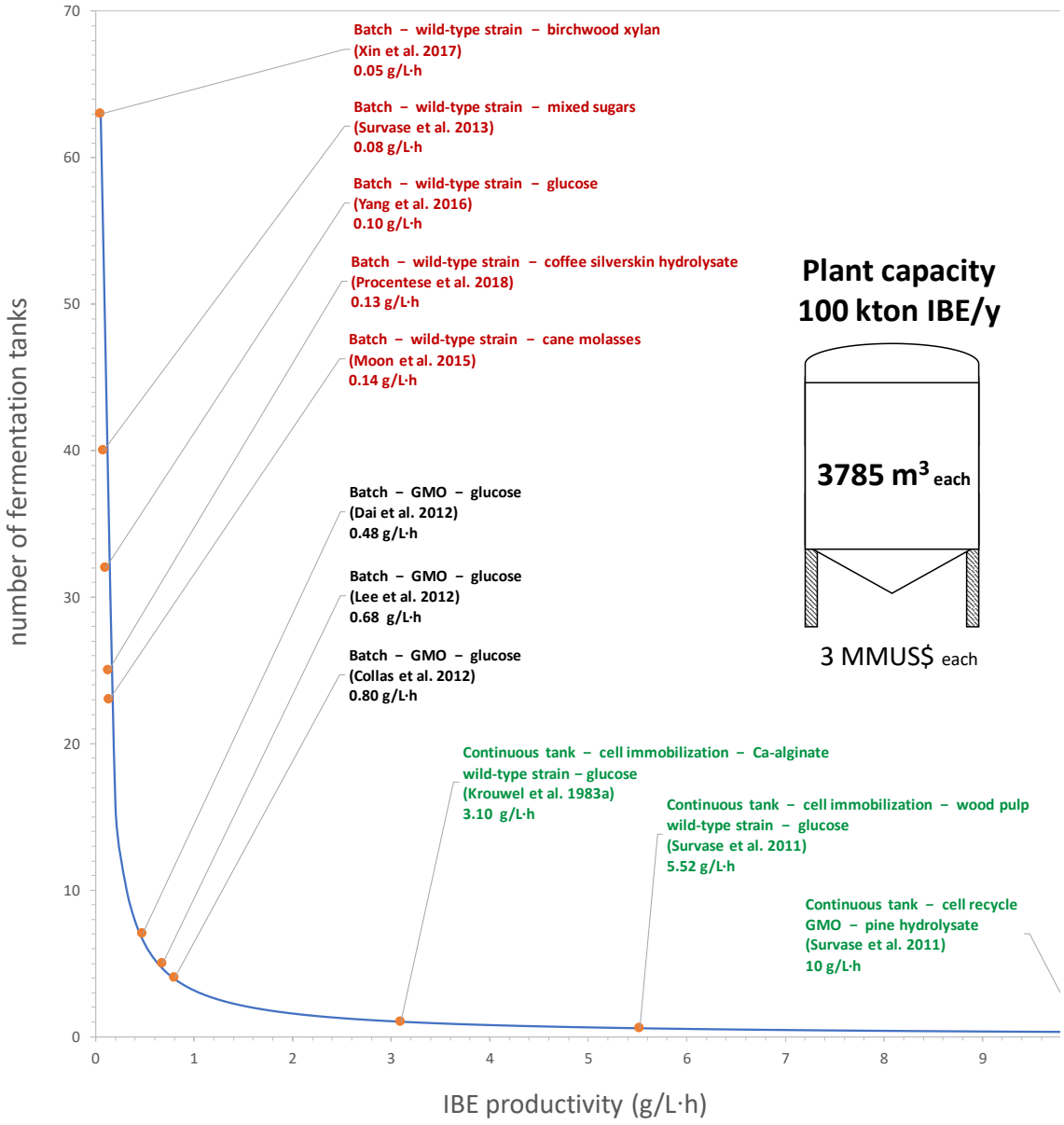


Figure 2.1.3 – Number of fermentation tanks as a function of IBE productivity for an IBE plant. Novel engineered IBE-producing *Clostridium* strains and cell retention systems can offer significant savings in capital investment of the fermentation unit.

In contrast, with engineered IBE-producing *Clostridium* strains, which have achieved productivities in the range 0.5−0.8 g/L·h (Table 2.1.2), the IBE plant would

have a more compact fermentation unit with 4 to 7 fermentation tanks. Furthermore, only one tank would suffice for a fermentation unit equipped with a cell retention system such as the continuous tank with membrane cell recycling developed by API (Survase et al., 2019). However, membrane fouling may be an issue (Mariano et al., 2015). Impressive results would also be achieved by other continuous tank fermentors with cells immobilized in Ca-alginate (Krouwel et al., 1983a) and wood pulp (Survase et al., 2011). Thus, gains in butanol concentration (resulting from a decrease in dilution rate) that are not accompanied by an IBE productivity lower than 0.30 g/L·h (which would result in 10 fermentors) may certainly be advantageous. In addition, any effort to increase IBE productivity above 1 g/L·h (region in which the curve in Figure 2.1.3 levels off) would have marginal effects on capital investment of an IBE plant.

2.1.5.2. *In-situ* product recovery

IBE productivity above 0.30 g/L·h has also been delivered by recently developed fermentation systems with integrated product recovery based on liquid-liquid extraction and gas stripping (Table 2.1.4). Furthermore, processing of more concentrated sugar solutions (40−90 g/L) has generally yielded product streams more concentrated in butanol (18−66 g/L). Product recovery has also improved sugar utilization in relation to control experiments without product recovery. In that regard, the IBE pilot plant developed by API is an interesting example (Survase et al., 2019). Besides fermenting the C6 and C5 sugar streams in separate continuous bioreactors, as discussed in section 2.1.4, sugar utilization in the pilot plant also increased due to integrated product recovery by liquid-liquid extraction (LLE). An LLE column was installed to recover solvents from the fermentation beer and the resulting IBE-depleted stream was sent back to the fermentors for further processing of unused sugars, nutrients, and metabolic intermediates (Figure 2.1.4a). Notably, xylose conversion improved from 63 to 81%.

But feeding of dilute beer to the continuous bioreactors was only possible because the beer was mixed with hydrolysates concentrated by evaporation. The cellulose hydrolysate was concentrated to a syrup containing 600 g/L sugars and the hemicellulose hydrolysate to 100 g/L sugars. However, this operation is costly and may offset gains in energy efficiency offered by liquid-liquid extraction. In fact, evaporation of hydrolysates to serve fermentation technologies with integrated product recovery has been found economically unfeasible (Pereira et al., 2018).

Table 2.1.4 – Integrated product recovery techniques applied to IBE fermentation.

Recovery technique	Carbon source	g/L	Operation mode	Strain	IBE yield (g/g sugar)	IBE productivity (g/L·h)	B (g/L)	I (g/L)	Reference
Adsorption (polymeric resin XAD 8)	Glucose	120	Repeated batch	*C. beijerinckii* LMD 27.6	-[a]	0.12	8.1	3.7	Groot and Luyben (1986)
Pervaporation	Glucose	33	Continuous (stirred tank)	*Clostridium spp.* DSM 2152[b]	-	-	-	-	van der Heiiden et al. (1989)
	Cane molasses	89	Continuous (column reactor)	*C. isopropylicum* IAM 19239	0.41	-	230[c]	-	Matsumura et al. (1992)
Gas stripping	Glucose and xylose	40/60	Batch	*C. beijerinckii* B593	0.32	0.29	-	-	Vrije et al. (2013)
	Glucose and xylose	40/60	Repeated Batch	*C. beijerinckii* B593	0.31	0.11	-	-	Vrije et al. (2013)
	Glucose and xylose	40/60	Continuous (stirred tank)	*C. beijerinckii* B593	0.30	1.30	-	-	Vrije et al. (2013)
	Glucose	60	Batch	*C. beijerinckii* B593	0.32	-	18.3	-	Pyrgakis et al. (2016)
	Glucose	80[d]	Fed-batch	C. *acetobutylicum* 824 PJC4BK(pIPA3-Cm2)	0.27	0.79	35.6[e]	-	Lee et al. (2012)
In situ liquid-liquid extraction[f] + gas stripping	Glucose	90	Batch	*C. beijerinckii* DSMZ 6423	0.35	-	16.5	-	Pérez-Bibbins et al. (2018)
Liquid-liquid extraction[g]	Glucose	50	Fed-batch	*C. beijerinckii* ATCC 6014	0.53	0.35	65.9[h]	-	Zhang et al. (2017)
In situ liquid-liquid extraction (biodiesel)	Glucose	90	Fed-batch	*Clostridium sp*. strain NJP7	0.41	0.22	25.6	5.2	Xin et al. (2017)
Liquid-liquid extraction + cell recycle[i]	Pine hydrolysate	60/30[i]	Continuous (stirred tank)	*C. acetobutylicum* DSM 792-ADH	0.37[j]	10.1[j]	7.7[k]	0.5[k]	Survase et al. (2019)

[a] not available;
[b] immobilized in Ca-alginate;
[c] concentration in the permeate;
[d] initial glucose concentration;
[e] produced from the consumption of 132.9 g/L of glucose along the fed-batch fermentation;
[f] sunflower oil (90% v/v) plus a C12 based Guerbet alcohol (2-Butyl −1-Octanol, 2B1O);
[g] fermentation broth is circulated through an external packed column reactor (cotton towel) followed by an external liquid-liquid extraction tank (aliphatic acids and oleyl alcohol);
[h] IB concentration in the extractant.
[i] pilot-scale continuous fermentation with membrane-assisted cell-recycling system; solvents were recovered from the cell-free beer by butyl butyrate in a continuous liquid-liquid extraction column; cellulosic (C6, 60 g/L) and hemicellulose (C5, 30 g/L) hydrolysates were fermented in separate tanks.
[j] average values (IBE + acetone) considering both C6 and C5 fermentations;
[k] only C6 fermentation.

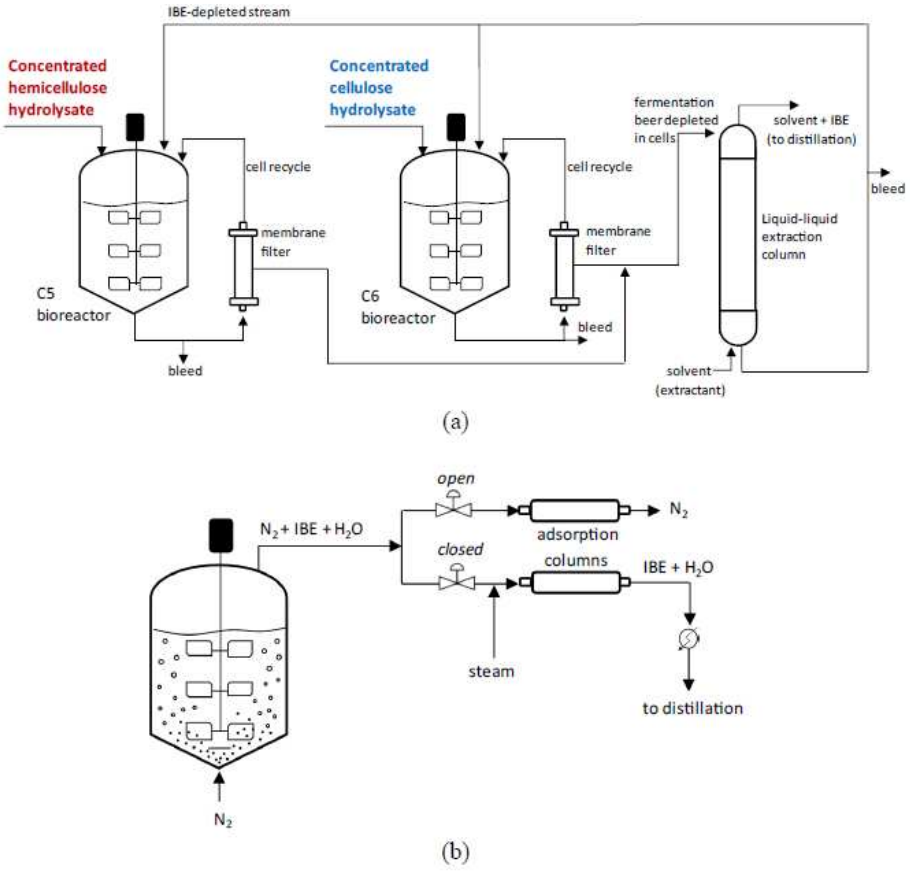


Figure 2.1.4 – Recently developed fermentation systems with integrated product recovery for IBE production. (a) Liquid-liquid extraction in the IBE pilot plant developed by American Process Inc. (Survase et al., 2019); (b) gas stripping technology and recovery of stripped solvents by adsorption onto carbon active (Pyrgakis et al., 2016).

Besides gains in productivity and butanol titer, studies on integrated recovery of IBE have also resulted in advances in the gas stripping technology. It is a relatively simple technique in which fermentation broth is flushed with N_2 or CO_2 and stripped solvents and water are recovered by condensation. Owing mainly to its simplicity, gas stripping has probably been the most studied technique to recover ABE from fermentation broth, with well-known contributions from The United States Department of Agriculture researcher Dr. Nasib Qureshi. One drawback of this technique is the low

removal rate, however. This problem motivated Vrije et al. (2013) to develop a strategy that combined repeated-batch operation with temperature elevation to improve the removal rate of IBE. At the end of each batch, temperature was raised to 70 ^{o}C before initiating a stripping session with N_2. Upon completion of product removal, ninety percent of the broth was removed, and fresh fermentation medium was added to restore the initial fermentation volume for the next batch. This strategy was efficient to improve the removal rate and resulted in a prolonged stable IBE culture. Moreover, the energy needed to heat fermentors in an IBE plant is expected to be offset by savings in downstream distillation. In another study, IBE removal rate by gas stripping was improved by associating this technique with liquid-liquid extraction (Pérez-Bibbins et al., 2018). This hybrid system allowed gas stripping to be operated intermittently and at lower N_2 flow rate (1.5 vvm against > 2.0 vvm when without LLE).

Another drawback of gas stripping, to which a solution was also proposed in IBE fermentation studies, is the difficulty in condensing stripped solvents. Condensation is challenging because of the presence of non-condensable gases (N_2 and fermentation gases CO_2 and H_2). In laboratory, condenser temperature is generally set at negative values. In industrial scale, however, the cost of condensing stripped solvents using cold utilities may be prohibitive. To solve this problem, adsorption onto carbon active (Sorbonorit® B3) was found to be a cost-effective mean to recover stripped solvents (Pyrgakis et al., 2016). More than 99% of stripped solvents can be recovered by this process, which consists of two adsorption columns operated alternately (adsorption/desorption) and in parallel so that continuous operation is possible (Figure 2.1.4b). The referred study demonstrated the economic advantages of adsorption over condensation, which are mainly given by energy efficiency and further gains from

energy integration. They also found that high energy cost makes condensation unsustainable.

Finally, with or without IBE recovery integrated with fermentation, IBE aqueous solutions must be dehydrated before commercialization (and separated if not sold as a fuel mixture). This downstream operation is generally touted as an advantage for IBE fermentation because the use of the mixture as a fuel would "eliminate the need for expensive recovery process and greatly improve the economic feasibility of IBE production" (Wang et al., 2018). However, while the ABE-water system forms two binary azeotropes (ethanol/water and butanol/water), in the IBE-water system a third binary azeotrope (isopropanol/water) further complicates dehydration (Díaz and Tost, 2017). This complication has motivated recent studies to develop conceptual designs of energy efficient downstream separation units for IBE processing based on distillation (Pyrgakis et al., 2016; Díaz and Tost, 2017). Furthermore, in case separation of isopropanol from the mixture is desired to sell this product in the chemical market, it implies further complications. Isopropanol and ethanol have similar boiling points and this characteristic prevents their separation by simple distillation. One possibility to circumvent this restriction is to use azeotropic distillation (US patent 5,338,411, "Separation of ethanol from isopropanol by azeotropic distillation"). Nonetheless, additional studies on downstream dehydration of IBE are needed to quantify potential gains in energy efficiency offered by integrated product recovery techniques.

2.1.6. Conclusions

Since early this decade, there has been growing interest in IBE fermentation to create a butanol fuel industry decoupled from acetone production. Important progress has been made in developing IBE-producing *Clostridium* strains more tolerant to butanol and highly productive fermentation processes. Although further work is needed

to prove the feasibility of these technologies at commercial scale, we expect they will serve as the basis for a future acetone-free butanol industry. We recommend that future progress should be supported by techno-economics and life-cycle assessment to identify promising feedstock-technology combinations and to quantify the carbon footprint of IBE fuel mixtures.

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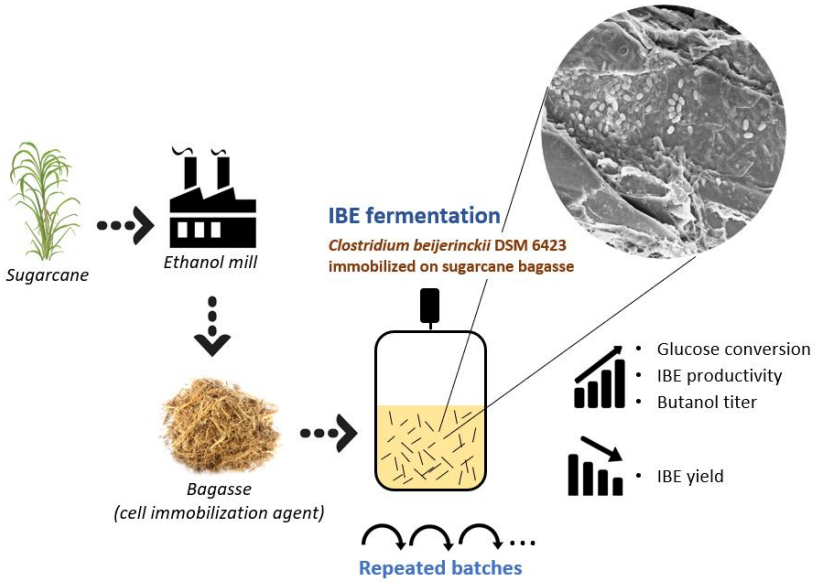
2.2. Isopropanol-butanol-ethanol (IBE) production in repeated-batch cultivation of *Clostridium beijerinckii* DSM 6423 immobilized on sugarcane bagasse

This section comprises the study of sugarcane bagasse as cell carrier for *Clostridium beijerinckii* DSM 6423 for IBE production. This section achieves objective 1 of Section 1.2.

Source:
VIEIRA, C.F.S., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P., 2019b. Isopropanol-butanol-ethanol (IBE) production in repeated-batch cultivation of *Clostridium beijerinckii* DSM 6423 immobilized on sugarcane bagasse. Fuel, 263, 116708.

GRAPHICAL ABSTRACT



ABSTRACT

The IBE mixture is a potential automotive fuel, and its production by *Clostridium beijerinckii* DSM 6423, the best known natural IBE producer, is hindered by low productivity and low butanol titer. To alleviate this problem, we cultivated *C. beijerinckii* DSM 6423 in repeated batches using sugarcane bagasse as a low-cost immobilization agent. Experiments were conducted in 250-mL bottles containing 150 mL P2 medium, glucose, and bagasse. In a fermentation with seven batch cycles (257 h) containing 7.5 g bagasse, glucose (60 g/L) conversion varied between 38% and 98%. In four of the batch cycles, IBE productivity was between 0.22 – 0.28 g/L·h, and butanol titer reached 6.7 – 8.6 g/L. In contrast, in a free-cell single-batch cultivation, glucose conversion was limited to 35%, IBE production was slower (0.13 g IBE/L·h), and butanol titer did not exceed 4.8 g/L. Despite the gains in productivity and butanol titer, further research is needed to elucidate the factors and mechanisms that caused IBE yield to decline during repeated-batch cultivation of *C. beijerinckii* DSM 6423.

Keywords: Biofuel, Butanol, Repeated batch, Cell immobilization, *Clostridium beijerinckii.*

2.2.1. Introduction

In recent years there has been a growing interest in the isopropanol-butanol-ethanol (IBE) fermentation as a means of providing a process alternative to develop an acetone-free butanol fuel industry (Vieira et al., 2019). This concern arises from the fact that in the conventional acetone-butanol-ethanol (ABE) fermentation, acetone is produced in amounts that correspond to roughly half of the butanol output. An ABE-based butanol fuel industry could thus cause the acetone market to fall out of balance. In contrast, the IBE mixture could be traded as a motor fuel (Li et al., 2019), thereby preventing the co-product from flooding the market. However, this proposed solution faces the challenge that natural IBE producers, such as *Clostridium beijerinckii* DSM 6423, are less efficient than ABE producers (Zhang et al., 2018).

While in the ABE process butanol is often obtained in the concentration range of 10 to 15 g/L, IBE-producing *Clostridium* species are less tolerant of butanol. As a result, butanol production in IBE batch fermentation is typically below 6 g/L (Vieira et al., 2019). Such low concentration can severely affect both the energy required to recover IBE from the fermentation broth and the wastewater footprint (Mariano and Maciel Filho, 2012; Grisales Diaz and Olivar Tost, 2017). Furthermore, IBE productivity is generally below 0.20 g/L·h, and it may cause IBE plants to demand an excessive number of fermentation tanks (Vieira et al. 2019). In response to these disadvantages, research has been conducted to engineer hyper IBE-producing strains derived from high-titer, high-yield, ABE-producing strains (Wang et al., 2018). Additionally, fermentors with cell immobilization (Zhang et al., 2016) and in-situ product recovery (Rochón et al., 2019) have also been proposed to improve productivity and butanol titer, respectively.

Past research on cell immobilization (mainly based on the simple technique of adsorption by passive adhesion) has enabled IBE productivity to exceed 1 g/L·h in continuous fermentation systems with dilution rate higher than 1 h^{-1} (Survase et al., 2011 and 2013; Bankar et al., 2014). However, the continuous reactors were not able to achieve the same level of butanol concentration and substrate conversion found in batch systems. To circumvent the problem of dilute product streams, the dilution rate of continuous bioreactors has been reduced in recent research. For example, in a column reactor operated at dilution rate of 0.085 h^{-1}, *C. beijerinckii optinoii* immobilized on ceramic Raschig rings delivered IBE productivity of 1 g/L·h and butanol concentration of 7.6 g/L (Yang et al., 2016). In other studies, the operation mode was changed to repeated batch to increase even more the butanol concentration. For instance, in a column reactor packed with cotton operated in repeated-batch mode, butanol concentration achieved 12 g/L and IBE productivity was 0.36 g/L·h (Zhang et al., 2016).

Besides operating parameters such as butanol titer and productivity, the cost effectiveness of immobilized cell reactors also depends on the cost of the cell carrier material. Such concern has prompted studies on the use of agricultural and wood-derived materials as immobilization carrier. In the ABE fermentation, for example, corn stalk bagasse (Cai et al., 2016) and sweet sorghum bagasse (Chang et al., 2014) allowed for stable butanol production in repeated-batch fermentation. Moreover, pretreatment of the sweet sorghum bagasse with NaOH enhanced the fermentation by providing the cells better accessibility to the rigid and crystalline structure of the lignocellulosic material. In the IBE fermentation, the potential of coconut fibers, wood chips, loofah sponge, and sugarcane bagasse to improve the performance of single-batch cultures of *C. beijerinckii* DSM 6423 has already been demonstrated (Survase et al., 2013).

Moreover, wood pulp was used to immobilize *C. beijerinckii* DSM 6423 in a continuous column reactor (Survase et al., 2011 and 2013).

C. beijerinckii DSM 6423 is the best known natural IBE producer, and its complete genome sequence was recently described by Gérando et al. (2018). However, IBE production by *C. beijerinckii* DSM 6423 in repeated batch mode has not yet been reported. So far only *C. beijerinckii* ATCC 6014, immobilized on cotton, has been studied for IBE production in repeated-batch fermentations (Zhang et al., 2016). Thus, the aim of this work was to evaluate the IBE production by *C. beijerinckii* DSM 6423 in repeated batches using sugarcane bagasse as immobilization agent. In the first step of this study we evaluated the effect of alkaline delignification and bagasse loading on the performance of single-batch fermentation. Repeated-batch fermentation using natural bagasse was then conducted under different conditions seeking operational stability.

2.2.2. Materials and methods

2.2.2.1. Microorganism and inoculum preparation

Clostridium beijerinckii DSM 6423 was used for IBE production. Culture stocks were maintained as spore suspensions in sterile distilled water at 4 °C. To prepare the inoculum, spores (400 µL) were heat-shocked for 10 min at 75 °C and then cooled on ice for 2 min. The heat-shocked spores were inoculated into 10 mL anoxic pre-sterilized TGY medium (g/L, 30 tryptone, 20 glucose, 10 yeast extract, 1 L-cysteine) and incubated in anaerobic chamber (COY Type A vinyl chamber) for 18 h at 35 °C. Ten mL of the actively growing culture were then transferred to 90 mL anoxic pre-sterilized TGY medium, and cells were cultivated under the same conditions for 5 h (fermentation inoculum).

2.2.2.2. Effect of delignification of bagasse on its use as cell immobilization carrier

Sugarcane bagasse was provided by Ester Sugar Mill S/A (Cosmópolis, SP,

Brazil). The bagasse was dried in open air under shade to a moisture content of 10% and then sieved. Bagasse particle sizes smaller than 3.35 mm were selected and used as cell immobilization carrier under three conditions: natural, water-washed, and alkaline pretreated. Alkaline pretreatment of the bagasse particles (2% w/v NaOH, 10% w/v solids loading, 121 ^{o}C, 1 h) was conducted in 500-mL flasks in autoclave. After pretreatment, the bagasse was washed with running water to remove unreacted pretreatment chemical and degradation products.

Single-batch fermentation culture of *C. beijerinckii* DSM 6423 was conducted in 250-mL screw-capped bottles (triplicate) containing 150 mL P2 medium, initial glucose concentration of 60 g/L, and sugarcane bagasse (solid-to-liquid ratio of 1:20 w/v, or 7.5 g bagasse in 150 mL culture medium). To prevent the bagasse from floating, it was wrapped in cotton gauze and placed inside perforated silicone tubes (11 mm internal diameter, 50 mm length; 10 mm round hole staggered; Figure 2.2.1). Each bottle contained eight tubes and a total of 2.0 g of gauze. The bottles containing fermentation medium (glucose and yeast extract solution) and the immobilization system were sterilized in autoclave (121 °C; 20 min) followed by cooling, addition of filter-sterilized (0.22 μm) P2 stock solutions (buffer, mineral, and vitamin solutions), and inoculation (10% v/v). The bottles were incubated still in anaerobic chamber for 55 hours at 35 °C. Control experiments were designed as follows: 50 g/L glucose fermentation without cell immobilization (Control I), 60 g/L glucose fermentation without cell immobilization (Control II), and 60 g/L glucose fermentation with cotton gauze placed inside the silicone tubes; however, without bagasse (Control III). Composition of the P2 medium was 1 g/L yeast extract, 1 % v/v buffer solution (g/L, 50 KH_2PO_4, 50 K_2HPO_4, 220 $C_2H_7NO_2$; pH 6.0), 1 % v/v mineral solution (g/L, 20 $MgSO_4.7H_2O$, 1 $MnSO_4.1H_2O$, 1 $FeSO_4.7H_2O$, 1 NaCl), and 1 % v/v vitamin solution (g/L, 0.1 $C_7H_7NO_2$, 0.1

$C_{12}H_{17}N_4OS^+$, 0.01 $C_{10}H_{16}N_2O_3S$).

Figure 2.1.1 – Bagasse floating during fermentation (left), and bagasse kept submerged wrapped in gauze inside silicone tubes (right).

2.2.2.3. Effect of bagasse loading on its use as cell immobilization carrier

In these experiments the single batch fermentation culture of *C. beijerinckii* DSM 6423 (conducted in triplicate as described in the previous section) contained unwashed natural bagasse in different solid-to-liquid ratios: 1:30; 1:50; and 1:75 w/v (respectively, 5.0; 3.0; and 2.0 g bagasse in 150 mL culture medium).

2.2.2.4. Repeated-batch fermentation with cell immobilization

Unwashed natural bagasse (kept submerged wrapped in gauze inside silicone tubes, as described in section 2.2.2.2) was used as cell immobilization carrier in the repeated-batch fermentation experiments. The repeated batches were conducted in 250-mL screw-capped bottles (triplicate, incubated in anaerobic chamber at 35 °C) and initiated with 150 mL P2 medium. Upon completion of the first and successive batches, 135 mL of the broth was removed, and fresh P2 medium was added to restore the initial

fermentation volume for the next batch. These experiments were conducted under different conditions regarding bagasse loading, initial sugar concentration, yeast extract (YE) concentration, and batch time (Table 2.2.1).

Table 2.2.1 – Process parameters of the repeated-batch fermentation experiment

	Experimental condition					
	I	II	III	IV	V	VI
Initial glucose (g/L)	60	60	60	60	40	40
Yeast extract (g/L)	1	1	1	0.5[1]	1	1
Bagasse-to-liquid ratio	1:20	1:75	1:20	1:20	1:20	1:75
Batch time (h)	55	55	36[2]	36[2]	48	48

[1] 1 g/L in the first two batches.

[2] 41.5 h in the first batch.

2.2.2.5. Analytical methods and calculations

The surface area and pore volume of the sugarcane bagasse (natural and alkaline pretreated) were measured by physical adsorption (BET and BJH methods, respectively) on a Micromeritics ASAP 2020 unit. Images of *C. beijerinckii* DSM 6423 cells adsorbed on the surface of natural and alkaline pretreated bagasse were generated by scanning electron microscope (SEM) (LEO Electron Microscopy 440i) equipped with an energy dispersive X-ray spectrometer (LEO Electron Microscopy 6070). For that purpose, bagasse samples from single-batch fermentation experiments were dried and covered with a thin layer of silver (200 Å) using a sputter coater (EMITECH K450).

Concentrations of glucose, IBE, acetic acid, and butyric acid were measured by high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity). The compounds were separated in a Bio-Rad Aminex® HPX-87H column (at 15 °C; 3 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL/min) and detected with refractive index detector (RID). IBE productivity (g/L·h) was calculated as amount of IBE produced (g/L) divided by fermentation time (h). IBE yield (g/g) was defined as the amount of IBE produced (g/L) per consumed glucose (g/L). Glucose conversion (%) was

calculated as amount of glucose consumed (g/L) divided by the initial glucose loading (g/L). Difference between means was statistically assessed by Tukey's test at 95% confidence interval using Minitab version 17 (Minitab Inc., State College, PA, USA). Data in tables are presented as mean ± standard deviation.

2.2.3. Results and discussion

2.2.3.1. Effect of delignification of bagasse on its use as cell immobilization carrier

In both natural and pretreated bagasse cases, cell immobilization had positive effects on the production of IBE by *C. beijerinckii* DSM 6423 in single-batch fermentation. Glucose conversion increased from 35% (control II without immobilization) to 72% (natural bagasse 1:20) and 83% (pretreated bagasse). Consequently, butanol concentration and IBE productivity also improved (Table 2.2.2). Notably, butanol concentration [7.1 (natural bagasse 1:20) and 8.3 (pretreated bagasse) g/L] was higher compared to values (< 6 g/L) commonly found in free-cell IBE batch fermentation (Vieira et al., 2019). *C. beijerinckii* DSM 6423 also produced more isopropanol when immobilized on both natural and pretreated bagasse. The increase in the I:B ratio may have been a strategy *C. beijerinckii* DSM 6423 found to alleviate product inhibition since isopropanol is less toxic than butanol (Gérando et al., 2016). As for the fermentation yield, the IBE yields were statistically the same in fermentations with cell immobilization [0.29 (natural bagasse 1:20) and 0.29 (pretreated bagasse)] and controls II (0.31) and III (0.35).

Table 2.2.2 – Effect of delignification of bagasse and bagasse loading on its use as cell immobilization carrier in single-batch cultivation of *C. beijerinckii* DSM 6423. Fermentation time was 55 h.

	Control I[1]	Control II[1]	Control III[2]	Natural bagasse[3] 1:20	NaOH-treated bagasse 1:20	Natural bagasse 1:30	Natural bagasse 1:50	Natural bagasse 1:75
Initial glucose (g/L)	47.6 ± 0.4	64.8 ± 2.1	60.8 ± 1.6	62.4 ± 1.5	60.0 ± 1.4	65.7 ± 1.2	66.0 ± 0.4	65.2 ± 3.0
Residual glucose (g/L)	29.3 ± 3.4	42.1 ± 2.1	33.7 ± 1.9	17.2 ± 0.4	10.3 ± 3.1	27.9 ± 0.8	30.0 ± 2.2	29.3 ± 1.2
Glucose conversion (%)	38 ± 7^{e}	35 ± 1^{e}	45 ± 4de	72 ± 1^{b}	83 ± 6^{a}	57 ± 0^{c}	55 ± 3^{cd}	55 ± 2^{c}
I (g/L)	1.7 ± 0.2^{c}	1.4 ± 0.3^{c}	3.1 ± 0.2^{b}	4.5 ± 0.1^{a}	4.4 ± 0.8^{a}	4.3 ± 0.3^{a}	3.7 ± 0.3^{ab}	3.8 ± 0.5^{ab}
B (g/L)	5.0 ± 0.2^{c}	4.8 ± 0.4^{c}	5.4 ± 0.2^{bc}	7.1 ± 0.1^{ab}	8.3 ± 1.2^{a}	7.5 ± 0.5^{a}	6.9 ± 0.6^{ab}	7.1 ± 0.9^{ab}
E (g/L)	0.4 ± 0.1^{d}	0.8 ± 0.2^{cd}	0.9 ± 0.1^{bcd}	1.5 ± 0.1^{ab}	1.4 ± 0.3^{abc}	1.7 ± 0.2^{a}	1.2 ± 0.2^{abc}	1.8 ± 0.5^{a}
Acetic acid (g/L)[4]	-1.0 ± 0.0	-1.8 ± 0.3	-1.1 ± 0.0	-1.2 ± 0.1	-1.3 ± 0.0	-1.5 ± 0.4	-1.4 ± 0.2	-1.4 ± 0.1
Butyric acid (g/L)	0.10 ± 0.00	0.10 ± 0.00	0.07 ± 0.01	0.09 ± 0.03	0.08 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.06 ± 0.00
IBE (g/L)	7.0 ± 0.4^{c}	7.0 ± 0.6^{c}	9.3 ± 0.4^{bc}	13.2 ± 0.2^{a}	14.2 ± 2.1^{a}	13.5 ± 0.7^{a}	11.7 ± 1.1^{ab}	12.7 ± 1.0^{a}
I:B:E mass ratio	4:14:1	1:4:1	3:6:1	3:5:1	3:6:1	3:4:1	3:6:1	2:4:1
IBE yield (g/g)	0.39 ± 0.05^{a}	0.31 ± 0.03^{ab}	0.35 ± 0.03^{ab}	0.29 ± 0.01^{b}	0.29 ± 0.04^{b}	0.36 ± 0.03^{ab}	0.33 ± 0.04^{ab}	0.35 ± 0.02^{ab}
IBE productivity (g/L·h)	0.15 ± 0.01^{c}	0.13 ± 0.01^{c}	0.17 ± 0.01^{bc}	0.24 ± 0.00^{a}	0.26 ± 0.04^{a}	0.25 ± 0.01^{a}	0.21 ± 0.02^{ab}	0.23 ± 0.02^{a}

Means and standard deviations followed by different letters are statistically different from each other (Tukey's test at 95% confidence interval).

[1] Without cell immobilization.

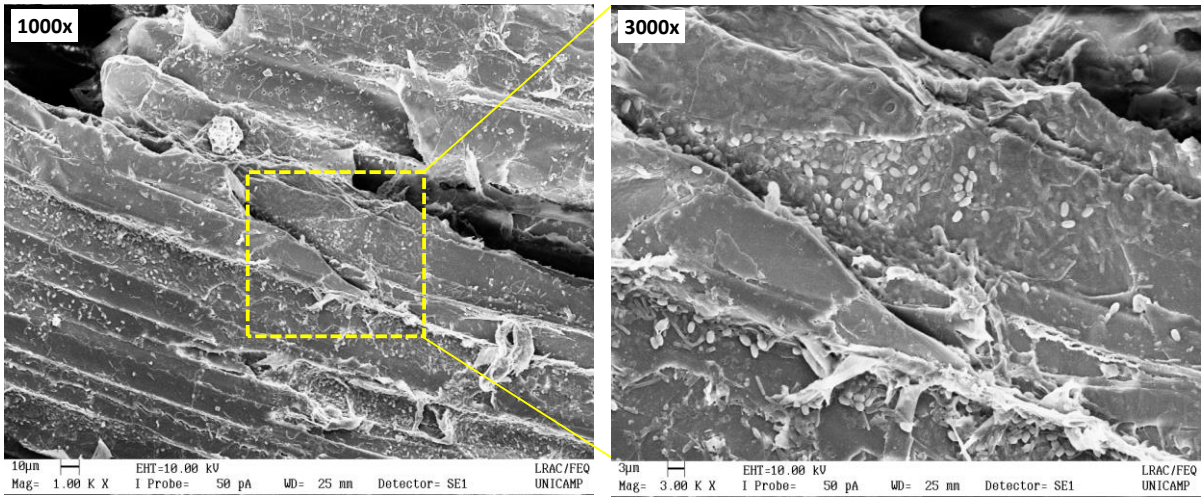
[2] Immobilization system (gauze + tubes) without bagasse.

[3] Unwashed bagasse. Washing the natural bagasse before fermentation had no significant difference (Tukey's test at 95% confidence interval).

[4] Negative concentration means that the final concentration was lower than the initial concentration.

SEM images of the bagasse at the end of the single-batch fermentation show that *C. beijerinckii* DSM 6423 cells adhered to the surface of the bagasse (Figure 2.2.2). Furthermore, the apparent higher density of cells observed in the SEM images of the pretreated bagasse may have resulted from an increase in surface area and porosity due to the alkaline treatment. Alkaline pretreatments are known for breaking the ester bonds between lignin and carbohydrates, decreasing the degree of polymerization and crystallinity and, thus, increasing the surface area (Behera et al. 2014; Cruz et al. 2018). The surface area of the bagasse increased by 27% [from 0.63 (natural bagasse) to 0.80 (pretreated bagasse) m^2/g], and the pore volume by 134% [from 0.001852 (natural bagasse) to 0.004345 (pretreated bagasse) cm^3/g]. This change in the morphological structure of the bagasse contributed to improve glucose conversion (Table 2.2.2). However, financial and environmental costs related to the alkaline pretreatment would only be justified if IBE production by *C. beijerinckii* DSM 6423 had improved. In fact, IBE production was statistically the same in the fermentation with natural bagasse and the fermentation with pretreated bagasse. Thus, in the next steps of our study we used unwashed natural bagasse.

Natural bagasse



NaOH-treated bagasse

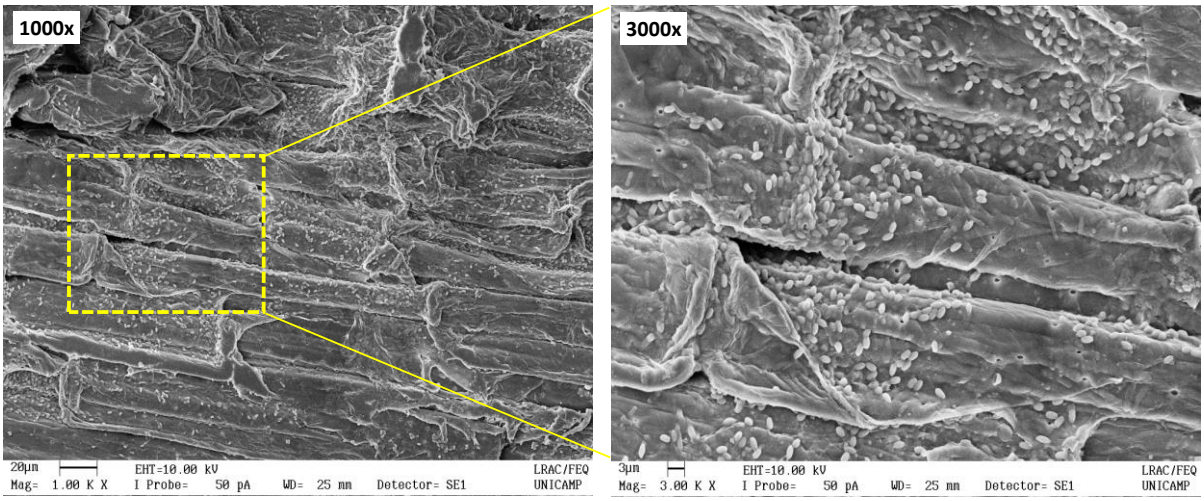


Figure 2.2.2 - SEM images of *C. beijerinckii* DSM 6423 cells adsorbed on the surface of natural and pretreated sugarcane bagasse at the end of the single-batch fermentation.

2.2.3.2. Effect of bagasse loading on its use as cell immobilization carrier

By decreasing the amount of bagasse in the single-batch fermentation (from 1:20 to 1:30, 1:50, and 1:75), IBE yield improved if compared with control I (Table 2.2.2). The IBE yield of 0.39 in control I (50-g glucose/L free-cell fermentation) was the highest found in this work, and it was statistically equal to the IBE yield (0.33 – 0.36) in fermentations with bagasse loading between 1:30 and 1:75. The improved IBE yield may have resulted from a lower cell growth, which can be inferred by the lower glucose consumption in the fermentations with lower bagasse loading. Interestingly, IBE

concentration and productivity were not affected in the cases with lower bagasse loading. Furthermore, isopropanol production was also favored under more dilute bagasse concentrations (Table 2.2.2).

2.2.3.3. Repeated-batch fermentation with cell immobilization

Under different experimental conditions, IBE production in repeated-batch fermentation was not stable and IBE yield generally decreased throughout the batches (Table 2.2.3). In experiments I (1:20 bagasse loading) and II (1:75 bagasse loading), sugar conversion dropped in the third batch. Consequently, butanol and isopropanol production declined. It was possible that the cells degenerated in the first two batches due to a long exposure to butanol. For this reason, in the next experiment (experiment III, 1:20 bagasse loading), the batch time was reduced from 55 to 36 h. As a result, during seven repeated batches (total fermentation time of 257 h) glucose conversion varied between 38% and 98%. In five batches (B1, B2, B5 to B7), glucose conversion was 1.5 to 2.7 times higher than the conversion (35%) found in the 60-g glucose/L free-cell fermentation (control II). Moreover, butanol concentration reached values between 6.7 and 8.6 g/L in the last three batches; consequently, IBE productivity (0.22 – 0.27 g/L·h) was higher compared with control II (0.13 g/L·h). However, after the third batch IBE yield decreased to values (0.15 – 0.18) below the yield in control II (0.31). Notably, isopropanol production was favored in the first two batches (as observed in the single-batch cultivation), and its production decreased in the following batches.

Table 2.2.3 – Performance of the repeated-batch cultivation of *C. beijerinckii* DSM 6423 under different experimental conditions.

Experimental condition	Performance parameter[1]	Repeated batches						
		B1	B2	B3	B4	B5	B6	B7
(I) 60 g/L glucose 1.0 g/L YE 1:20 55h	Initial glucose (g/L)	64.0 ± 1.0	62.2 ± 2.1	58.3 ± 2.0	-	-	-	-
	Glucose conversion (%)	62 ± 2*	71 ± 3*	26 ± 8	-	-	-	-
	I (g/L)	5.5 ± 0.1*	5.4 ± 0.1*	1.6 ± 0.5	-	-	-	-
	B (g/L)	6.2 ± 0.2*	5.9 ± 0.2*	2.6 ± 0.6**	-	-	-	-
	E (g/L)	0.3 ± 0.0**	0.3 ± 0.0**	0.3 ± 0.1**	-	-	-	-
	IBE (g/L)	12.0 ± 0.3*	11.6 ± 0.4*	4.5 ± 1.1**	-	-	-	-
	Acetic acid (g/L)	-1.8 ± 0.0	-1.4 ± 0.0	0.6 ± 0.5	-	-	-	-
	Butyric acid (g/L)	0.42 ± 0.02	0.21 ± 0.07	0.06 ± 0.00	-	-	-	-
	I:B:E mass ratio	18:21:1	18:20:1	5:9:1	-	-	-	-
	IBE yield (g/g)	0.30 ± 0.02	0.26 ± 0.01	0.30 ± 0.06	-	-	-	-
	IBE productivity (g/L·h)	0.22 ± 0.01*	0.21 ± 0.01*	0.08 ± 0.02**	-	-	-	-
(II) 60 g/L glucose 1.0 g/L YE 1:75 55h	Initial glucose (g/L)	64.9 ± 0.7	63.5 ± 2.9	60.2 ± 1.3	-	-	-	-
	Glucose conversion (%)	54 ± 3*	33 ± 5	8 ± 1**	-	-	-	-
	I (g/L)	4.3 ± 0.0*	1.3 ± 0.5	0.8 ± 0.1**	-	-	-	-
	B (g/L)	5.4 ± 0.1*	1.9 ± 0.7**	1.1 ± 0.2**	-	-	-	-
	E (g/L)	0.5 ± 0.1	0.2 ± 0.0**	0.2 ± 0.1**	-	-	-	-
	IBE (g/L)	10.2 ± 0.0*	3.4 ± 0.2**	2.2 ± 0.3**	-	-	-	-
	Acetic acid (g/L)	1.7 ± 0.0	-0.2 ± 0.1	0.2 ± 0.0	-	-	-	-
	Butyric acid (g/L)	0.07 ± 0.00	0.36 ± 0.01	0.29 ± 0.03	-	-	-	-
	I:B:E mass ratio	9:11:1	7:10:1	4:5:1	-	-	-	-
	IBE yield (g/g)	0.29 ± 0.02	0.16 ± 0.04**	0.46 ± 0.01*	-	-	-	-
	IBE productivity (g/L·h)	0.19 ± 0.00*	0.06 ± 0.02**	0.04 ± 0.00**	-	-	-	-
(III) 60 g/L glucose 1.0 g/L YE 1:20 36 h	Initial glucose (g/L)	58.8 ± 1.4	59.3 ± 2.2	61.3 ± 2.1	61.8 ± 1.0	54.9 ± 1.4	63.9 ± 0.6	56.4 ± 0.3
	Glucose conversion (%)	61 ± 1*	52 ± 4*	38 ± 20	60 ± 32	98 ± 2*	80 ± 17*	94 ± 6*
	I (g/L)	3.5 ± 0.0*	2.1 ± 0.0*	0.8 ± 0.6	0.3 ± 0.2**	0.6 ± 0.2**	0.6 ± 0.1**	0.6 ± 0.1**
	B (g/L)	7.1 ± 0.0*	4.5 ± 0.2	3.7 ± 1.5	5.2 ± 3.9	8.6 ± 1.0*	7.0 ± 1.1*	6.7 ± 1.1*
	E (g/L)	1.2 ± 0.1*	0.2± 0.2**	0.2 ± 0.1**	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.1
	IBE (g/L)	11.8 ± 0.1*	6.8 ± 0.1	4.7 ± 0.9**	6.0 ± 3.9	9.6 ± 1.3*	8.1 ± 1.3	7.9 ± 1.3
	Acetic acid (g/L)	-0.9 ± 0.1	-1.2 ± 0.1	-0.5 ± 0.3	-0.7 ± 0.5	-1.2 ± 0.3	-1.0 ± 0.1	-1.1 ± 0.0
	Butyric acid (g/L)	0.17 ± 0.00	0.23 ± 0.01	1.03 ± 0.08	1.51 ± 0.32	2.15 ± 0.16	0.85 ± 0.03	1.87 ± 0.09
	I:B:E mass ratio	3:6:1	11:23:1	4:18:1	1:10:1	2:21:1	1:14:1	1:11:1
	IBE yield (g/g)	0.32 ± 0.02	0.22 ± 0.01**	0.23 ± 0.08	0.16 ± 0.03**	0.18 ± 0.02**	0.17 ± 0.06**	0.15 ± 0.03**
	IBE productivity (g/L·h)	0.28 ± 0.00*	0.16 ± 0.04	0.13 ± 0.03	0.17 ± 0.11	0.27 ± 0.04*	0.23 ± 0.04*	0.22 ± 0.04*
(IV) 60 g/L glucose 0.5 g/L YE 1:20 36 h	Initial glucose (g/L)	57.9 ± 4.4	60.7 ± 1.7	58.0 ± 1.2	58.2 ± 1.0	60.3 ± 2.4	59.1 ± 1.8	61.7 ± 1.2
	Glucose conversion (%)	83 ± 7*	54 ± 4*	75 ± 9*	28 ± 22	27 ± 23	33 ± 12	50 ± 5*
	I (g/L)	4.9 ± 0.4*	3.0 ± 1.0*	2.9 ± 0.3*	1.3 ± 0.1	1.4 ± 1.6	1.0 ± 0.8	2.3 ± 0.2*
	B (g/L)	6.5 ± 0.1*	3.6 ± 0.4**	5.0 ± 1.0	1.5 ± 1.3**	1.8 ± 1.8**	1.8 ± 0.8**	2.8 ± 0.1**
	E (g/L)	2.9 ± 0.9*	5.2 ± 1.8*	5.2 ± 0.1*	1.1 ± 1.0	1.9 ± 1.6	1.9 ± 1.3	2.8 ± 1.0*
	IBE (g/L)	14.3 ± 1.2*	11.8 ± 1.7*	13.1 ± 0.9*	3.9 ± 3.4	5.1 ± 4.5	4.7 ± 2.9	7.9 ± 1.3
	Acetic acid (g/L)	-0.7 ± 0.3	-0.6 ± 0.1	-0.4 ± 0.1	0.0 ± 0.1	0.5 ± 1.0	0.4 ± 0.0	-1.4 ± 0.1
	Butyric acid (g/L)	0.08 ± 0.00	0.45 ± 0.03	0.33 ± 0.01	1.66 ± 0.09	0.07 ± 0.03	0.53 ± 0.11	1.19 ± 0.25
	I:B:E mass ratio	1.7:2.2:1	0.6:0.7:1	0.6:1.0:1	1.2:1.4:1	0.7:1.0:1	0.5:1.0:1	0.8:1.0:1
	IBE yield (g/g)	0.30 ± 0.01	0.37 ± 0.07	0.31 ± 0.02	0.19 ± 0.11	0.24 ± 0.14	0.23 ± 0.07	0.25 ± 0.02
	IBE productivity (g/L·h)	0.34 ± 0.03*	0.33 ± 0.04*	0.37 ± 0.04*	0.11 ± 0.09	0.14 ± 0.13	0.13 ± 0.08	0.22 ± 0.04*
(V) 40 g/L glucose 1.0 g/L YE 1:20 48h	Initial glucose (g/L)	43.3 ± 0.4	34.0 ± 2.0	32.5 ± 0.3	35.8 ± 2.9	40.3 ± 2.2	37.7 ± 2.2	43.1 ± 3.5
	Glucose conversion (%)	100 ± 0*	95 ± 8*	82 ± 19*	49 ± 28	57 ± 23	47 ± 39	49 ± 19
	I (g/L)	1.4 ± 0.5	1.6 ± 0.1	1.6 ± 0.6	0.6 ± 1.0	1.1 ± 0.9	1.3 ± 1.9	1.2 ± 0.9
	B (g/L)	6.6 ± 0.1*	5.3 ± 0.6	4.7 ± 1.1	2.9 ± 2.2	1.1 ± 1.1**	1.7 ± 2.6**	1.5 ± 1.2**
	E (g/L)	4.3± 0.2*	3.1 ± 0.4*	2.8 ± 0.9*	1.6 ± 1.5	0.1 ± 0.5	0.4 ± 0.3	0.8 ± 0.3
	IBE (g/L)	12.3 ± 0.4*	10.0 ± 1.0*	9.1 ± 2.5	5.1 ± 4.7	2.3 ± 1.7**	3.4 ± 4.7	3.5 ± 1.8**
	Acetic acid (g/L)	-1.2 ± 0.0	-0.4 ± 0.1	-0.4 ± 0.3	-0.2 ± 0.4	-0.3 ± 0.4	-0.1 ± 0.6	-0.5 ± 0.2
	Butyric acid (g/L)	0.11 ± 0.00	0.12 ± 0.01	0.46 ± 0.06	0.32 ± 0.02	0.01 ± 0.00	0.11 ± 0.03	0.00 ± 0.00
	I:B:E mass ratio	0.3:1.5:1	0.5:1.7:1	0.6:1.7:1	0.4:1.8:1	11:11:1	3.3:4.3:1	1.5:1.9:1
	IBE yield (g/g)	0.28 ± 0.01**	0.31 ± 0.03	0.34 ± 0.02	0.26 ± 0.09	0.10 ± 0.04**	0.12 ± 0.12**	0.16 ± 0.03**
	IBE productivity (g/L·h)	0.26 ± 0.01*	0.21 ± 0.02*	0.19 ± 0.05	0.11 ± 0.10	0.05 ± 0.04**	0.07 ± 0.10	0.07 ± 0.04**
(VI) 40 g/L glucose 1.0 g/L YE 1:75 48h	Initial glucose (g/L)	43.2 ± 0.1	41.0 ± 0.3	42.5 ± 3.9	40.6 ± 3.0	42.3 ± 0.5	43.1 ± 1.9	47.7 ± 1.5
	Glucose conversion (%)	94 ± 6*	50 ± 30	69 ± 26	28 ± 10	15± 2**	28 ± 8	22 ± 5**
	I (g/L)	1.7 ± 0.1	1.0 ± 0.8	1.5 ± 0.8	0.0 ± 0.0**	0.4 ± 0.3**	0.2 ± 0.1**	0.3 ± 0.1**
	B (g/L)	6.5 ± 0.3*	3.7 ± 2.0	4.5 ± 2.6	0.4 ± 0.4**	0.5 ± 0.4**	0.3 ± 0.1**	0.5 ± 0.2**
	E (g/L)	3.7 ± 0.2*	2.1 ± 1.5	2.4 ± 1.3	0.2 ± 0.2	0.0 ± 0.1**	0.1 ± 0.0**	0.1± 0.1**
	IBE (g/L)	11.9 ± 0.5*	6.8 ± 4.2	8.4 ± 4.7	0.6 ± 0.7**	0.9 ± 0.8**	0.6 ± 0.2**	0.9 ± 0.3**
	Acetic acid (g/L)	-0.2 ± 1.7	-0.4 ± 0.3	-0.5 ± 0.4	-0.4 ± 0.7	-0.2 ± 0.6	-0.2 ± 0.1	-0.2 ± 0.1
	Butyric acid (g/L)	0.06 ± 0.02	0.24 ± 0.06	0.29 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	I:B:E mass ratio	0.5:1.8:1	0.5:1.8:1	0.6:1.9:1	0:2.0:1	-	2:3:1	3:5:1
	IBE yield (g/g)	0.29 ± 0.01**	0.34 ± 0.09	0.27 ± 0.10	0.05 ± 0.05**	0.14 ± 0.10**	0.04 ± 0.01**	0.10 ± 0.04**
	IBE productivity (g/L·h)	0.25 ± 0.01*	0.14 ± 0.09	0.17 ± 0.11	0.01 ± 0.01**	0.02 ± 0.02**	0.01 ± 0.00**	0.02 ± 0.01**

[1] Calculated based on subtracting the final concentration from the initial concentration in each batch cycle (except initial glucose concentration). Negative concentration of acetic acid means a decrease in concentration compared to initial value.

*, ** Means (glucose conversion, I, B, E, IBE, IBE yield, IBE productivity) are statistically higher (*) or lower (**) compared with control experiment (Tukey's test at 95% confidence interval). Experiments I to IV were compared with control II, and experiments V and VI were compared with control I. Performance of control experiments is presented in Table 2.

One possible reason for the decrease in IBE yield was an increase in cell growth. In experiment III, a visual thin biofilm layer was formed probably as a response to the relative high butanol concentration (6.7 – 8.6 g/L) in the last three batches. Biofilms can provide a diffusive barrier against butanol (Qureshi et al. 2005), and improved tolerance to butanol due to biofilm formation was also observed in previous works on IBE (Survase et al. 2013; Yang et al. 2016; Zhang et al. 2016) and ABE (Liu et al. 2014) fermentation. In the fifth batch of experiment III, glucose conversion was high as 98%, but IBE yield was 0.18. A second reason may be a change in the metabolism of *C. beijerinckii* DSM 6423 given that the I:B ratio declined from 0.5 to 0.1 during the repeated cultivations. Modification of metabolism and decline of fermentation performance was also observed when *C. acetobutylicum* CICC 8012, an ABE producer, was immobilized on sugarcane bagasse and cultivated during seven repeated batches (Liu et al. 2019). Nonetheless, this possible change in metabolism did not affect the re-assimilation of the intermediate products acetic and butyric acids. In most of the batch cycles in experiment III, the final concentration of acetic acid was lower than the initial concentration (Table 2.2.3). Furthermore, even if the butyric acid produced in batches 4 to 7 (0.85 – 2.15 g/L) were converted into butanol, IBE yield would still be statistically lower than the IBE yield in control II. Therefore, the decline of IBE yield cannot be attributed to carbons being used in acid production.

Attempting to solve the problem of yield decline, we tested two strategies. In the first strategy, we varied the amount of yeast extract used in the fermentation of experiment III. As such, in experiment IV we cut in half the concentration of yeast extract as of the third batch seeking to slow down cell growth. As a result, the IBE yield improved compared with experiment III. In the seven batches of experiment IV, the IBE yield was statistically equal to the yield in control II. But on the other hand, under YE-

limited condition butanol production decreased to values (1.5 – 2.8 g/L in batches 4 to 7) below that found in control II (4.8 g/L). The second strategy consisted in decreasing the glucose loading because the IBE yield in the 50-g glucose/L free-cell fermentation was high as 0.39 (Control I, Table 2.2.2 presented in section 2.2.3.1). However, under both more concentrated (1:20 in experiment VI) and more dilute (1:75 in experiment VII) bagasse loading conditions, butanol production decreased after the third batch. Thus, butanol production and IBE productivity were lower compared with the free-cell cultivation (control I). Interestingly, in experiments IV to VI ethanol production was favored in the first three batches, achieving the highest value in all experiments (5.2 g/L in the second batch of experiment IV). The reasons for this unexpected elevated ethanol production are unknown at this stage.

Despite not solving the problem of yield decline, the gains in fermentation performance offered by the conditions of experiment III are important. Glucose conversion increased significantly compared with free-cell batch cultivation (in 5 out 7 batches conversion was higher than in control II). Furthermore, the gain in productivity from 0.13 (control II without immobilization) to 0.22 – 0.28 g/L·h would also decrease the number of fermentation tanks required by an IBE plant. According to a relationship between number of fermentation tanks and IBE productivity (Vieira et al., 2019), the number of fermentation tanks (3785 m^3 each tank) would decrease from 21 to at least 15 tanks in case cell immobilization with bagasse were used in a 100-kt/a IBE plant. However, further studies are needed to find ways to minimize the decline of products yield during the repeated-batch cultivation of *C. beijerinckii* DSM 6423.

New studies have been undertaken, for example, to characterize the biofilm formed by *C. beijerinckii* DSM 6423 during continuous fermentation (Carrie et al. 2019; Hocq et al. 2019). Furthermore, had the change in the metabolism of *C.*

beijerinckii DSM 6423 been caused by the exposure of the cells to butanol concentrations higher than 6 g/L, we speculate that in-situ product recovery (e.g. gas stripping, vacuum fermentation) would help to solve this problem. Despite that, losses of sugar due to biofilm growth could be counterbalanced to some extent by reusing the bagasse for lignocellulosic sugar production. In this operation, carbohydrates present in the extracellular polymeric substances of the biofilm could be hydrolyzed to sugars and used for IBE production.

2.2.4. Conclusions

Repeated-batch cultivation of *C. beijerinckii* DSM 6423 immobilized on natural sugarcane bagasse is an efficient strategy to improve sugar utilization, butanol titer, and productivity. However, likely changes in the metabolism of *C. beijerinckii* DSM 6423 led to loss of product yield. Future studies are needed to elucidate the factors and mechanisms responsible for the changes in the metabolism of *C. beijerinckii* DSM 6423 in repeated-batch cultivation so that process design can be used to help solving the problem.

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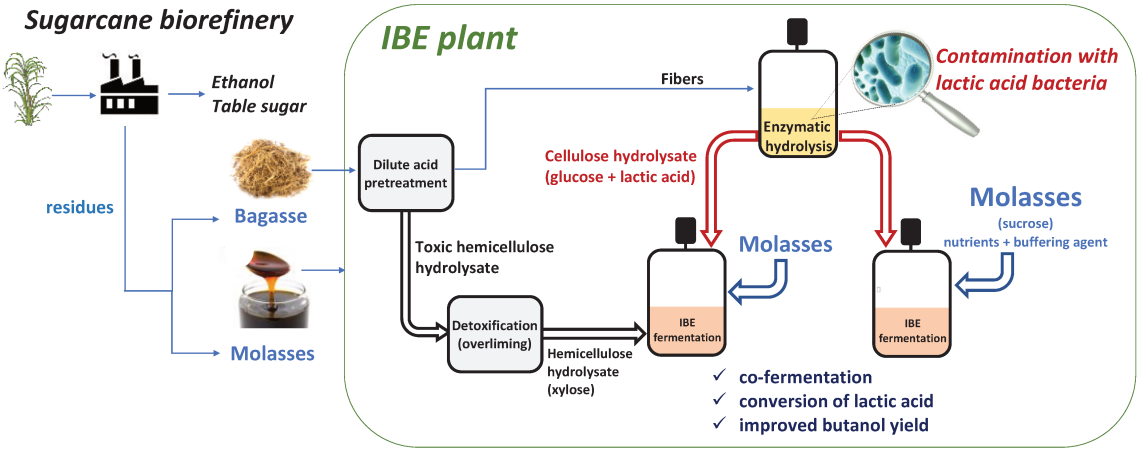
2.3. Sugarcane bagasse hydrolysates as feedstock to produce the Isopropanol-Butanol-Ethanol (IBE) fuel mixture: effect of lactic acid derived from microbial contamination on *Clostridium beijerinckii* DSM 6423

This section comprises the study of second-generation substrates, i.e. sugarcane bagasse cellulose and hemicellulose hydrolysates, to produce IBE. This section achieves objective 4 of Section 1.2.

Source:
VIEIRA, C.F.S., CODOGNO, M.C., MAUGERI FILHO, F., MACIEL FILHO, R., MARIANO, A.P. Sugarcane bagasse hydrolysates as feedstock to produce the isopropanol-butanol-ethanol fuel mixture: Effect of lactic acid derived from microbial contamination on *Clostridium beijerinckii* DSM 6423. Bioresour. Technol. 319, 124140.

GRAPHICAL ABSTRACT



ABSTRACT

Enzymatic hydrolysis of lignocellulose under industrial conditions is prone to contamination by lactic acid bacteria, and in this study, a cellulose hydrolysate produced from dilute-acid pretreated sugarcane bagasse contained 13 g/L lactic acid and was used for IBE production by *Clostridium beijerinckii* DSM 6423. In fermentation of the cellulose hydrolysate supplemented with sugarcane molasses for nutrients and buffering of the medium (40 g/L total sugar), 92% of the lactic acid was consumed, and the butanol yield was as high as 0.28 (7.9 g/L butanol), suggesting that lactic acid was preferentially metabolized to butanol. When the hydrolysate was mixed with a detoxified bagasse hemicellulose hydrolysate and supplemented with molasses (35 g/L total sugar), the culture was able to exhaust glucose and utilized sucrose (by 38%), xylose (31%), and lactic acid (70%). Overall, this study shows that *C. beijerinckii* DSM 6423 can co-ferment first- and second-generation sugars while consuming lactic acid.

Keywords: Cellulose hydrolysate, microbial contamination, lactic acid, molasses, *Clostridium beijerinckii* DSM 6423.

2.3.1. Introduction

The demand for advanced biofuels has raised the interest in renewable butanol, and its production via the isopropanol–ethanol–butanol (IBE) fermentation has an important advantage over the historical acetone–ethanol–butanol (ABE) fermentation. In the IBE fermentation, solventogenic clostridia convert the acetone they produce into isopropanol, which can also be used as an automotive fuel. In contrast, acetone can corrode engine rubber parts and has poor fuel properties (Li et al., 2019). Furthermore, if butanol is produced via ABE fermentation in a multi-million-ton scale per annum to meet biofuel mandates, the associated acetone output (about half of that of butanol) could flood the chemical market. To avoid this problem, the attention given to the IBE fermentation has been increasing (Vieira et al., 2019), advancing the research not only on new microbes (Wang et al., 2018) and bioreactors (Rochón et al., 2020; Dantas et al., 2020) but also on effective strategies to convert lignocellulosic sugars into IBE (Survase et al., 2019).

IBE-producing clostridia can metabolize several types of lignocellulosic sugars, including xylose (Survase et al., 2013), and yet their ability to ferment lignocellulosic hydrolysates varies depending on whether the hydrolysate is of cellulose or hemicellulose. Previous studies, although limited to wood feedstock, have demonstrated that the conversion of hemicellulose hydrolysate into IBE is challenged by lignocellulose-derived microbial inhibitory compounds (e.g., furans, organic acids, and phenolic compounds) formed under harsh hydrolysis conditions (Bankar et al., 2014; Survase et al., 2019). In contrast, cellulose hydrolysates are generally produced by enzymatic hydrolysis under mild conditions (45–50°C and pH 4.8–5.0), and virtually no microbial inhibitory compounds are formed from the degradation of lignocellulose. This characteristic allowed crude (non-detoxified) cellulose hydrolysates of cassava bagasse

(Zhang et al., 2016), coffee silverskin (Procentese et al., 2018), pine (Survase et al., 2019), and eucalyptus (Cebreiros et al., 2019) to be fermented to IBE. Due to their good fermentability, cellulose hydrolysates have been proposed to be the initial substrate in the fed-bach culture of solventogenic clostridia, to which hemicellulose hydrolysates with a high level of toxic chemicals can be fed when the growth of the culture is vigorous (Qureshi et al., 2018).

However, the literature on the production of lignocellulosic sugars suggests that the use of cellulose hydrolysates to produce IBE on an industrial scale can be challenged by acids derived from microbial contamination. Significant contamination may occur because of the long batch time (up to five days) needed to hydrolyze cellulose under mild temperature and non-sterile conditions. This problem has been observed in pilot-scale cellulosic ethanol facilities, in which cellulose hydrolysates produced under semi-sterile conditions were contaminated by lactic acid bacteria (detected in several input streams to hydrolysis, including pretreated fiber, enzyme storage tank, and transfer lines). The contaminating microorganisms consumed sugar and produced lactic acid (up to 25 g/L) that was detrimental to fermentation (Schell et al., 2007; Serate et al., 2015). The problem was solved by adding antibiotics during hydrolysis, a strategy that has also been employed in laboratory-scale experiments (Pietrobon et al., 2011; Awan et al., 2013). However, the use of antibiotics cannot only be economically and environmentally prohibitive (Schell et al., 2007), but it may also affect solventogenic clostridia.

Because of these restrictions, a company (American Process Inc., USA) that has been developing technology for the IBE fermentation proposed the use of sulfur dioxide (the chemical they use to pretreat the biomass feedstock) to deter microbial contamination during the enzymatic hydrolysis step (Retsina et al., 2016). However, as

also mentioned in their patent, most of the sulfur remaining in the cellulose hydrolysate must be removed before fermentation because sulfur-containing compounds inhibit clostridia (He and Chen, 2020). By contrast, previous research on ABE fermentation suggests that enzymatic hydrolysis under non-sterile conditions in IBE plants may be viable because some ABE-producing clostridia can consume lactic acid and tolerate concentrations as high as 7 to 10 g/L (Oshiro et al., 2010; Yoshida et al., 2012; Zhou et al., 2018). Thus, although some sugar may be consumed to produce lactic acid, the lactic acid can eventually be converted into butanol. However, the effect of lactic acid on IBE-producing clostridia has not yet been investigated.

In this study, sugarcane bagasse cellulose hydrolysate produced in a pilot plant, containing 13 g/L lactic acid derived from microbial contamination, was used for IBE production. The aim, therefore, was to assess whether *C. beijerinckii* DSM 6423 – the best known IBE-producing *Clostridium* species – can consume lactic acid in fermentations having cellulose hydrolysate as the sole source of lignocellulosic sugar (glucose) and mixed with a bagasse hemicellulose hydrolysate containing xylose and lignocellulose-derived microbial inhibitory compounds. Moreover, to avoid the use of synthetic nutrients (such as diammonium phosphate and urea) and related carbon emission, this study also assessed whether sugarcane molasses (a by-product of the sugar industry rich in sucrose) can be used as a source of nutrients and buffering capacity. The efficiency of molasses was benchmarked against laboratory-grade nutrients and buffer, and the order of sugar preference by the DSM 6423 strain was also investigated.

2.3.2. Materials and methods

2.3.2.1. Feedstock and preparation of hydrolysates

Sugarcane blackstrap molasses and sugarcane bagasse (hereafter referred to as molasses and bagasse, respectively) were supplied by Ester Sugar Mill S/A (Cosmópolis, SP, Brazil). The molasses contained 606 g/L sugar (g/L, 450 sucrose, 87 glucose, and 69 fructose) and was used in the fermentation experiments without hydrolysis treatment. The bagasse was shipped directly to the Brazilian Biorenewables National Laboratory (LNBR) (CNPEM, Campinas, Brazil) and processed in a cellulosic ethanol pilot plant (lnbr.cnpem.br/en/facilities). The bagasse was cleaned by vibrating sieves (Multideck Multivibro, Brazil), and 20 dry kg of the bagasse was pretreated (145 oC, 12 min) with 200 L dilute sulfuric acid (0.5% v/v) in a 350-L Hastelloy C-276 mixing reactor (POPE Scientific Inc., Saukville, USA). The resulting slurry was filtered (140-L Hastelloy C-22 Nutsche Filter, USA), and the filtrate consisted of the hemicellulose hydrolysate (HH). The dilute-acid pretreated bagasse (12 dry kg) was washed in a centrifuge (Hastelloy C-22 VTC 400/200 Ferrum, Switzerland) six times with water and fed to the enzymatic hydrolysis reactor (the 350-L mixing reactor). Solids loading was 12% w/v, the initial pH 4.8 (adjusted using 2 M H_2SO_4 and NaOH solutions), and cellulase concentration 15 FPU/g dry solids (390 mL Cellic Ctec 2 Novozymes, 464 FPU/mL). Enzymatic hydrolysis was conducted at 50 °C, 70 rpm for 72 h under non-sterile conditions. The resulting slurry was filtered in the Nutsche filter, and the filtrate consisted of the cellulose hydrolysate (CH). The hemicellulose and cellulose hydrolysates were stored in non-sterile 20-L plastic jugs for five days at room temperature (~30 °C) before being delivered to the laboratory for analysis.

The pH of the HH was adjusted to 6.5 using 2 M NaOH, and some of the HH was detoxified by overliming with calcium hydroxide (Qureshi et al., 2010). The pH of the over-limed HH was adjusted to 6.5 using concentrated H_2SO_4. The hydrolysates were

not checked for microbial contamination and were kept frozen (-5 °C) before use in fermentations.

2.3.2.2. Microorganism and inoculum preparation

Clostridium beijerinckii DSM 6423 procured from the German Collection of Microorganisms and Cell Culture, Braunschweig, Germany (DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen) was used for IBE production. Details on culture maintenance (spore stock solution) and inoculum preparation using tryptone-glucose-yeast extract (TGY) medium are given by Vieira et al. (2020).

2.3.2.3. IBE fermentation

Batch fermentation experiments were organized into three groups (Table 2.3.1).

Table 2.3.1 – Fermentation media and their composition

	Medium		Composition
Group 1 Fermentation of cellulose hydrolysate	1	CH + P2	Cellulose hydrolysate (CH) + P2 stock solutions
	2	CH + low M	Cellulose hydrolysate (CH) + molasses (6 g/L initial sucrose)
	3	CH + high M	Cellulose hydrolysate (CH) + molasses (12 g/L initial sucrose)
	4	P2 + LA	40 g/L glucose + P2 stock solutions[1] + 20 g/L lactic acid[2]
Group 2 Fermentation of mixture of hydrolysates	5	HH	Hemicellulose hydrolysate (HH) + P2 stock solutions
	6	Detox-HH	Over-limed HH + P2 stock solutions
	7	Detox-HH + CH	75% Over-limed HH + 25% CH (%v/v) + P2 stock solutions
	8	Detox-HH + CH + M	75% Over-limed HH + 25% CH (%v/v) + molasses
Group 3 Sugar preference: Fermentation of molasses and laboratory-grade sugars	9	M	Diluted molasses
	10	M + P2	Diluted molasses + P2 stock solutions
	11	S	Sucrose + P2 stock solutions
	12	X	Xylose + P2 stock solutions
	13	S + X	Sucrose + Xylose + P2 stock solutions
	14	S + G + F + X	Sucrose + Glucose + Fructose + Xylose + P2 stock solutions

[1]1 g yeast extract/L, minerals, vitamins, and phosphate and ammonium acetate buffer (Vieira et al., 2020)
[2]85% L-(+)-lactic acid solution in H_2O, CAS number 79-33-4, ACS reagent grade.

In the first group (fermentation of cellulose hydrolysate), CH was supplemented with either P2 stock solutions [1 g yeast extract/L, minerals, vitamins, and phosphate and ammonium acetate buffer (pH 6.0) as described by Vieira et al. (2020)] (medium 1) or molasses at two concentration levels, resulting in 6 and 12 g/L initial sucrose concentration (media 2 and 3). Additionally, the *C. beijerinckii* DSM 6423 culture was challenged with 20 g/L lactic acid (medium 4).

In the second group (fermentation of a mixture of hydrolysates), the initial experiments assessed the fermentability of (i) the crude and over-limed HH (media 5 and 6) (ii) and a mixture of the over-limed HH with CH supplemented with either P2 stock solutions or molasses (media 7 and 8). The third group (fermentation of molasses and laboratory-grade sugars) was devised to investigate the sugar preference by the DSM 6423 strain using fermentation media 9 to 14 containing (i) molasses and (ii) laboratory-grade sugars (sucrose, glucose, fructose, and xylose).

The initial pH of fermentation media 1 to 4 was adjusted to 6.0 by adding 2 M NaOH, and the initial pH (6.0 – 6.2) of media 5 to 14 was not adjusted. The fermentation media were sterilized in an autoclave (121 °C; 20 min) followed by cooling and overnight storage in the anaerobic chamber (COY Type A vinyl chamber, atmosphere of 96% N_2 and 4% H_2), and inoculation with *C. beijerinckii* DSM 6423 (10% v/v). Batch culture (150 mL) without pH control of *C. beijerinckii* DSM 6423 was conducted in 250-mL screw-capped bottles (triplicate) incubated statically at 35 °C in the anaerobic chamber.

2.3.2.4. Analytical methods and calculations

Concentration of sugars (sucrose, glucose, fructose, and xylose), organic acids [L-(+)-lactic, acetic, and butyric acids], and solvents (isopropanol, n-butanol, and ethanol) was measured by high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity) using a Bio-Rad Aminex® HPX-87H column (at 15 °C, 3 mM H_2SO_4 as mobile phase, at a flow rate of 0.5 mL/min) and a refractive index detector (RID). Furfural and hydroxymethylfurfural (HMF) were separated using a Nova-Pak C18 HPLC column (30 °C; 88:11:1 v/v water:acetonitrile:acetic acid solution as mobile phase at a flow rate of 0.8 mL/min) and detected by UV–Vis at 280 nm. Phenolic compounds were measured by a colorimetric assay (Ainsworth and Gillespie, 2007).

IBE productivity (g/L·h) was calculated as the amount of IBE produced (g/L) divided by fermentation time (h). IBE yield (g/g) is the amount of IBE produced (g/L) per reducing sugar (1.05 × sucrose + glucose + fructose + xylose) consumed (g/L). Sugar conversion (%) was calculated as the amount of reducing sugar consumed (g/L) divided by the initial concentration of reducing sugar (g/L). The buffer capacity (Van Slyke's buffer index, β) of fermentation media 7 and 8 (Table 2.3.1) was determined by titration with HCl (0.1 M) and calculated as the amount of acid (mol HCl/dm^3 solution titrated) needed to lower the pH of the medium by one unit. The difference between means was statistically assessed by Tukey's test ($p < 0.05$) using the web-based open-access tool Astatsa Online Web Statistical Calculators (Navendu Vasavada, astatsa.com). Data in tables are presented as mean ± standard deviation.

2.3.3. Results and discussion

2.3.3.1. Fermentation of cellulose hydrolysate

When cultivated in the cellulose hydrolysate, *C. beijerinckii* DSM 6423 was able to tolerate 13 g/L lactic acid and consumed most (~90%) of the lactic acid (Figure 2.3.1).

Furthermore, the substitution of the P2 stock solutions (minerals and buffer) by molasses at a low concentration level (medium 2: CH + low M) did not affect the fermentation in terms of IBE concentration, yield, and productivity (Table 2.3.3). Sugars were co-fermented, and sucrose (6.2 g/L or 16% of the initial TRS) and fructose (1.9 g/L) were exhausted in 54 h whereas 61% of the glucose was consumed in 96 h. Interestingly, regardless of the type of supplementation (P2 or molasses at a low concentration level), the final butanol concentration was 7.1–7.9 g/L, and the butanol yield was 0.28–0.30. These values of butanol yield are significantly higher than previously reported values obtained when the DSM 6423 strain was cultivated without

the presence of lactic acid. For instance, the average butanol yield in 42 fermentations in defined glucose P2 medium (batch operation with and without cell immobilization) was 0.14 (Vieira et al., 2020). Similarly, the average butanol yield was 0.17 in seven free-cell continuous fermentation runs using the glucose P2 medium (Survase et al., 2011). In other studies with non-defined media, the butanol yield was 0.19 when the DSM 6423 strain was cultivated in sugarcane and sweet sorghum juices (Rochón et al., 2019 and 2020), and 0.15 when cultivated in eucalyptus cellulose hydrolysate supplemented with P2 stock solutions (Cebreiros et al., 2019).

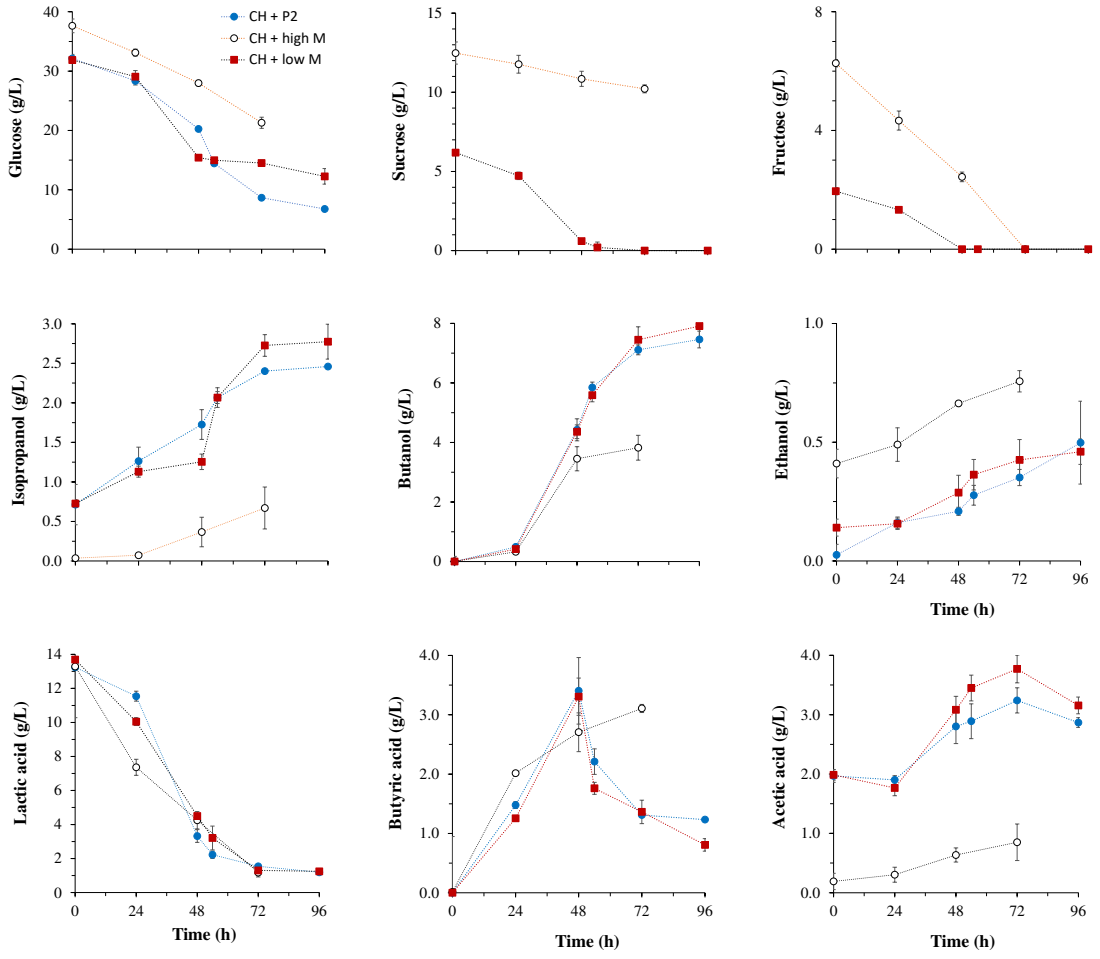


Figure 2.3.1. Concentration of sugars, IBE, and lactic, butyric, and acetic acids during fermentation of cellulose hydrolysate supplanted with (i) P2 stock solutions (medium 1) and (ii) molasses at two concentration levels (media 2 and 3).

The comparison with previous works suggests that the lactic acid found in the cellulose hydrolysate was mostly converted into butanol by *C. beijerinckii* DSM 6423. That is to say that during the conversion of lactate into pyruvate by lactate dehydrogenase and then into acetyl-CoA (Oshiro et al., 2010; Zhou et al., 2018), the resulting carbon flow from acetyl-CoA [the central intermediate in the branched fermentation pathways in solventogenic clostridia, Jones et al. (1986)] was preferentially driven to butanol. This hypothesis is corroborated by the observations of Oshiro et al. (2010), who demonstrated using isotope analysis that the ABE-producing *C. saccharoperbutylacetonicum* N1-4 converts lactic acid mainly into butanol and, secondarily, into CO_2, acetone, and ethanol.

Another performance indicator that needs to be highlighted is the IBE productivity, which was statistically the same regardless of the type of supplementation (fermentations CH + P2 and CH + low [molasses]; Table 2.3.3), and similar to the productivity (0.15 g/L·h IBE) achieved by the DSM 6423 strain when cultivated in defined medium without lactic acid (Vieira et al., 2020). Linked to productivity, the lag phase of *C. beijerinckii* DSM 6423 did not increase due to the lactic acid found in the cellulose hydrolysate. This is interesting because the lag phase of the ABE-producing *C. saccharoperbutylacetonicum* N1-4 increased when cultivated in a glucose medium containing 5 g/L lactic acid (initial pH was 6.5) (Zhou et al., 2018). Under these conditions, the lag phase lasted 120 h, and the N1-4 strain was able to produce 7.0 g/L butanol in the following 72 h (when 7.5 g/L lactic acid was present, no cell activity of the N1-4 strain was observed). As for *C. beijerinckii* DSM 6423, when challenged with 20 g/L lactic acid (fermentation medium 4), no activity was observed during 120 h. This result suggests that the minimum inhibitory concentration (MIC) of lactic acid to *C. beijerinckii* DSM 6423 is between 14 and 20 g/L.

In contrast to the good results presented above, the addition of molasses at a high concentration level (medium 3: CH + high M) to the cellulose hydrolysate negatively affected the fermentation (Figure 2.3.1). Sucrose (12 g/L or 23% of the initial TRS) was only partially consumed (by 18%), and the IBE yield and productivity were lower compared with media 1 (CH + P2) and 2 (CH + low [molasses]) (Table 2.3.3). The poorer performance can be attributed to glucose-induced carbon catabolite repression (Ezeji et al., 2014). Indeed, to improve the fermentability of sucrose, molasses was usually treated with invertase to convert sucrose into glucose and fructose in commercial ABE fermentation processes operated between the 1930s and 1950s (Shaheen et al., 2000; Moon et al., 2015). Therefore, to avoid additional costs related to treating the molasses – or the repression of sucrose utilization by glucose if the molasses is not treated, the amount of molasses to be added to the cellulose hydrolysate has to be the minimum required to supply enough nutrients and buffering capacity.

Another important factor to enable the production of IBE from a cellulose hydrolysate containing lactic acid is the pH. The pH of the cellulose hydrolysate increased from 4.9 to 5.5 by adding the P2 stock solutions, and when the pH was not adjusted to 6.0 using 2 M NaOH, *C. beijerinckii* DSM 6423 was not able to produce IBE from the cellulose hydrolysate. The pKa of lactic acid is 3.86, and by increasing the pH from 5.5 to 6.0, the amount of undissociated lactic acid decreased by 35% (from 23 to 15 mM, calculated using the Henderson–Hasselbalch equation). Thus, less undissociated lactic acid was available to enter the cell cytosol, thereby decreasing the inhibitory effect caused by the dissociation of weak acids at neutral intracellular pH (Qi et al., 2017).

2.3.3.2. Fermentation of a mixture of hydrolysates

The hemicellulose hydrolysate (HH) contained lignocellulose-derived microbial inhibitory compounds (Table 2.3.2) that prevented *C. beijerinckii* DSM 6423 from fermenting either the crude HH (fermentation medium 5) or the detoxified hemicellulose hydrolysate (medium 6).

Table 2.3.2 – Concentration (g/L) of sugars and lignocellulose-derived microbial inhibitory compounds in the sugarcane bagasse hydrolysates.

	Cellulose hydrolysate	Hemicellulose hydrolysate	Hemicellulose hydrolysate (over-limed)
Glucose	32.0	6.5	5.0
Xylose	1.7	23.3	21.2
Lactic Acid	13.5	-	-
Acetic Acid	-	3.7	3.3
Furfural	-	0.5	0.2
HMF	-	0.6	0.3
Phenolic Compounds	-	1.3	0.97

It is unknown at this stage which compounds most affected the culture; nevertheless, IBE production was enabled by mixing the over-limed hemicellulose hydrolysate with the cellulose hydrolysate in a volume ratio of 75:25 (HH:CH). By contrast, the mixture of crude hemicellulose hydrolysate with cellulose hydrolysate was not fermented. Note that detoxification by overliming results in sugar loss (Table 2.3.2) and generates gypsum (calcium sulfate), a solid residue. Because of these problems, other detoxification techniques such as ammonia conditioning (Humbird et al., 2011) have been considered more adequate to treat hemicellulose hydrolysates.

By mixing the over-limed hemicellulose hydrolysate containing 26.2 g/L total sugar with the cellulose hydrolysate (33.7 g/L total sugar) (Table 2.3.3), the sugar concentration in the mixture was 24 g/L. Molasses was then used to increase the sugar concentration to 72 g/L (34 g/L sucrose or 49% of the initial TRS) and as a substitute for the laboratory-grade nutrients (P2 stock solutions). The addition of molasses to the

mixture of hydrolysates significantly enhanced the production of IBE by *C. beijerinckii* DSM 6423. The IBE concentration (8.3 g/L) and productivity (0.15 g/L·h) were significantly higher than the concentration (4.8 g/L IBE) and productivity (0.09 g/L·h IBE) obtained in the respective fermentation supplemented with P2 stock solutions. Furthermore, regardless of the type of supplementation, most of the lactic acid was consumed (by 76–79%), and the butanol yield (0.22) was higher compared with the typical values (0.14 – 0.19) reported in the literature (presented in Section 2.3.3.1).

Table 2.3.3 – Performance of the batch cultivation of *C. beijerinckii* DSM 6423 in the cellulose hydrolysate supplemented with either P2 stock solutions (medium 1) or sugarcane molasses at two concentration levels (media 2 and 3).

	CH + P2		**CH + low [molasses]**		**CH + high [molasses]**
Initial TRS (g/L)	32.1 ± 0.7		40.3 ± 0.5		57.0 ± 1.8
Fermentation time (h)	72	96	72	96	72
TRS conversion (%)	73.1 ± 0.5^{ab}	78.9 ± 1.1^{a}	64.0 ± 1.5^{c}	69.6 ± 3.2^{bc}	43.8 ± 3.3^{d}
Sucrose conversion (%)	-	-	100 ± 0.0	100 ± 0.0	17.9 ± 4.7
Fructose conversion (%)	-	-	100 ± 0.0	100 ± 0.0	100 ± 0.0
Glucose conversion (%)	73.1 ± 0.5	78.9 ± 1.1	54.4 ± 1.8	61.5 ± 4.0	43.3 ± 4.8
LA conversion (%)	88.3 ± 0.7^{a}	91.0 ± 0.2^{a}	88.8 ± 1.5^{a}	91.7 ± 0.7^{a}	91.1 ± 2.4^{a}
I (g/L)	1.7 ± 0.3^{a}	1.7 ± 0.1^{a}	2.0 ± 0.2^{a}	2.1 ± 0.3^{a}	0.6 ± 0.3^{b}
B (g/L)	7.1 ± 0.2^{a}	7.5 ± 0.3^{a}	7.4 ± 0.4^{a}	7.9 ± 0.4^{a}	3.8 ± 0.5^{b}
E (g/L)	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.3 ± 0.1^{a}
IBE (g/L)	9.1 ± 0.5^{a}	9.7 ± 0.3^{a}	9.7 ± 0.4^{a}	10.3 ± 0.5^{a}	4.8 ± 0.6^{b}
B yield (g/g)	0.30 ± 0.00^{a}	0.29 ± 0.01^{a}	0.29 ± 0.01^{a}	0.28 ± 0.02^{a}	0.15 ± 0.03^{b}
IBE yield (g/g)	0.39 ± 0.02^{a}	0.38 ± 0.01^{a}	0.38 ± 0.02^{a}	0.37 ± 0.02^{a}	0.19 ± 0.03^{b}
IBE productivity (g/L·h)	0.13 ± 0.01^{ab}	0.10 ± 0.00^{c}	0.14 ± 0.01^{a}	0.11 ± 0.01^{bc}	0.07 ± 0.01^{d}

Means and standard deviations followed by different letters are statistically different from each other (Tukey's test at 95% confidence interval).

A possible explanation for the benefits of the supplementation with molasses is that it increased the pH buffering capacity of the medium. The salt content in molasses is generally 2 to 8% (Clarke, 2003; Lino et al., 2018), and the buffering capacity of the mixture with molasses (0.049 mol/dm^3) was 30% higher compared with that of the respective mixture with P2 stock solutions (0.037 mol/dm^3). This increase in the buffering capacity may have attenuated the inhibitory effect of the weak organic acids

present in the fermentation. Indeed, other buffering agents such as $CaCO_3$, KOH (Qi et al., 2017; Wang et al., 2019), and biochar (Sun et al., 2020) have been employed to alleviate the inhibition of ABE-producing clostridia by lignocellulose-derived organic acids. Furthermore, the observed benefits offered by the molasses suggests that salts from molasses did not inhibit *C. beijerinckii* DSM 6423 during the fermentation of hydrolysates.

Despite the gains in IBE production offered by the addition of molasses to the hydrolysate mixtures, the amount of residual sugar (~50 g/L) in the fermentation is by no means satisfactory (Table 2.3.4). Moreover, while glucose and fructose were preferentially consumed (66 and 58 %, respectively), sucrose and xylose were consumed to a much lesser extent (17 and 32 %, respectively). To reduce the loss of sugar, the fermentation of the mixture of hydrolysates was repeated with approximately four times less molasses, resulting in an initial total reducing sugar (TRS) concentration of 35 g/L (g/L, 8.7 sucrose + 13.5 glucose + 1.6 fructose + 10.3 xylose), as presented as follows.

In the fermentation of the mixture containing less molasses, glucose was preferentially consumed and exhausted in 64 h (Figure 2.3.2). Meanwhile, *C. beijerinckii* DSM 6423 was able to co-ferment sucrose and xylose; however, they were consumed (38 and 31%, respectively) to a lesser extent than glucose. By contrast, fructose was not utilized. As for the acids, the DSM 6423 strain consumed 70% of the lactic acid and 60% of the acetic acid. The final IBE concentration was 7.6 g/L IBE (75% of the IBE was produced in 24 h), and the IBE yield was 0.40. Moreover, the butanol yield (0.29) was statistically different from the fermentations containing more molasses (medium 8). Therefore, the decrease in the amount of molasses in the fermentation allowed better utilization of the sugars (especially the glucose from the

hydrolysates, which accounted for approximately 85% of the glucose in the medium) improving the butanol yield.

Table 2.3.4 – Performance of the batch cultivation of *C. beijerinckii* DSM 6423 in the mixture of cellulose and hemicellulose hydrolysates supplemented with either P2 stock solutions (medium 6) or sugarcane molasses (medium 7). The fermentation time was 54 h.

	Detox-HH + CH (P2 stock solutions)	**Detox-HH + CH + M (molasses)**
Initial TRS (g/L)	24.1 ± 0.3	71.8 ± 2.0
Residual TRS (g/L)	8.0 ± 0.7	46.9 ± 1.6
TRS conversion (%)	66.8 ± 3.0[a]	34.6 ± 3.9[b]
Initial sucrose (g/L)	-	33.7 ± 0.6
Residual sucrose (g/L)	-	27.9 ± 0.9
Initial fructose (g/L)	-	6.7 ± 0.8
Residual fructose (g/L)	-	2.8 ± 0.2
Initial glucose (g/L)	12.0 ± 0.2	18 ± 0.4
Residual glucose (g/L)	0.0 ± 0.0	6.1 ± 0.5
Initial xylose (g/L)	11.0 ± 2.0	11.5 ± 0.5
Residual xylose (g/L)	8.0 ± 0.7	8.7 ± 0.4
Initial LA (g/L)	3.8 ± 0.1	3.5 ± 0.3
Final LA (g/L)	0.8 ± 0.1	0.8 ± 0.1
LA conversion (%)	78.7 ± 2.3[a]	75.8 ± 3.0[a]
I (g/L)	1.0 ± 0.0[b]	2.5 ± 0.1[a]
B (g/L)	3.6 ± 0.3[b]	5.1 ± 0.0[a]
E (g/L)	0.2 ± 0.1[b]	0.7 ± 0.1[a]
IBE (g/L)	4.8 ± 0.3[b]	8.3 ± 0.1[a]
Butyric acid (g/L)	0.9 ± 0.2	0.9 ± 0.4
Acetic acid (g/L)[1]	-0.1 ± 0.1	-0.3 ± 0.1
B yield (g/g)	0.22 ± 0.00[a]	0.21 ± 0.03[a]
IBE yield (g/g)	0.30 ± 0.01[a]	0.34 ± 0.04[a]
IBE productivity (g/L·h)	0.09 ± 0.01[b]	0.15 ± 0.01[a]

LA: lactic acid; TRS: total reducing sugar.
Means and standard deviations followed by different letters are statistically different from each other (Tukey's test at 95% confidence interval).
[1]Negative concentration means that the final concentration was lower than the initial concentration.

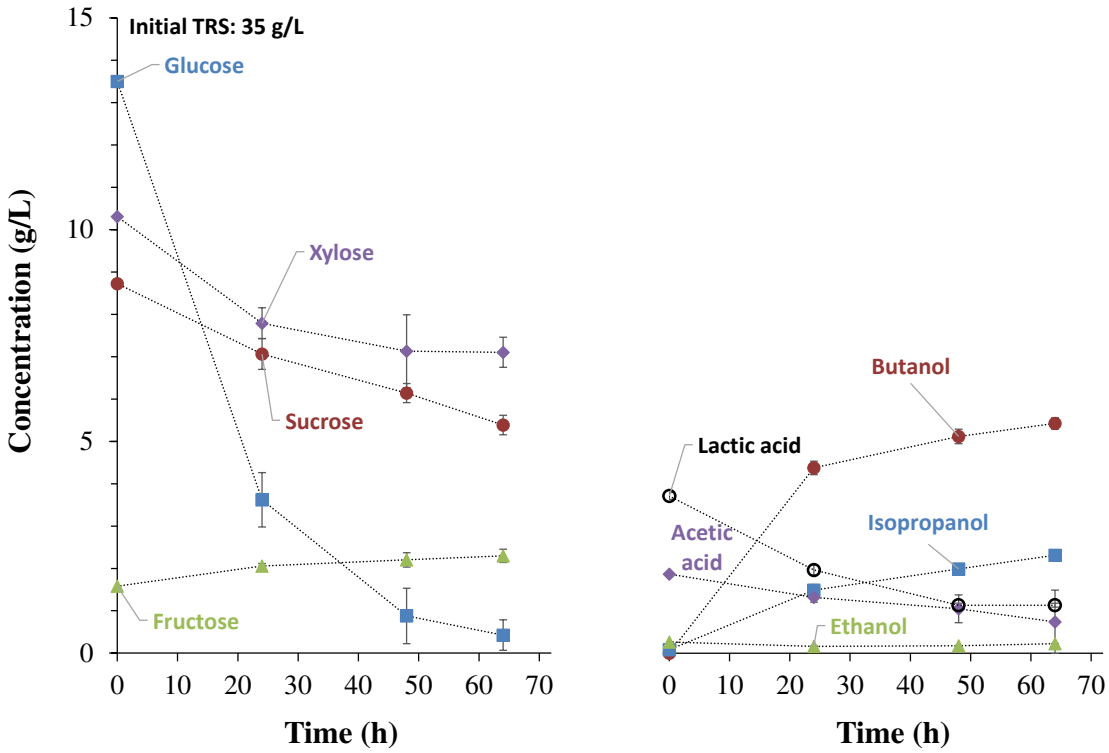


Figure 2.3.2. Concentration of sugars, IBE, acetic acid, and lactic acid during fermentation of the mixture containing hemicellulose hydrolysate (HH), cellulose hydrolysate (CH), and molasses. The volume ratio of hydrolysates was 75:25 (HH:CH), and the initial total reducing sugar (TRS) was 35 g/L.

2.3.3.4. Sugar preference

The preferential uptake of glucose by the DSM 6423 strain was also observed during the fermentation of molasses (media 9 and 10) and cultivation in defined media (media 11, 13, 14). In the fermentation of diluted molasses (71 g/L initial TRS), *C. beijerinckii* DSM 6423 was able to exhaust glucose and fructose while the conversion of sucrose was incomplete (47–49%) (Table 2.3.5). The resulting IBE (0.16–0.18) and butanol (0.09–0.11) yields were markedly lower compared with the experiments presented in the previous sections. Furthermore, differently from previous studies that used P2 stock solutions to enhance the IBE yield from sugarcane molasses (Moon et al., 2015) and sugarcane juice (Rochón et al., 2019), this effect was not observed in the present study (fermentation medium M + P2) (Table 2.3.5).

Table 2.3.5 – Fermentation of molasses (medium 9) and molasses supplemented with P2 stock solutions (medium 10). The fermentation time was 54 h.

	M	**M + P2**
Initial TRS (g/L)	71.3 ± 2.7	77.1 ± 7.3
Residual TRS (g/L)	28.7 ± 0.5	29.7 ± 1.1
TRS conversion (%)	59.6 ± 2.0[a]	61.2 ± 4.4[a]
Initial sucrose (g/L)	52.3 ± 2.3	55.6 ± 4.4
Residual sucrose (g/L)	27.3 ± 0.5	28.3 ± 1.1
Initial fructose (g/L)	6.3 ± 0.1	7.2 ± 1.1
Residual fructose (g/L)	0.0 ± 0.0	0.0 ± 0.0
Initial glucose (g/L)	9.9 ± 0.3	11.4 ± 1.7
Residual glucose (g/L)	0.0 ± 0.0	0.0 ± 0.0
I (g/L)	1.8 ± 0.1[a]	1.7 ± 0.1[a]
B (g/L)	4.2 ± 0.6[a]	4.3 ± 0.1[a]
E (g/L)	1.6 ± 0.2[a]	1.5 ± 0.2[a]
IBE (g/L)	7.6 ± 0.3[a]	7.5 ± 0.2[a]
Butyric acid (g/L)	1.4 ± 0.1	1.3 ± 0.2
Acetic acid[1] (g/L)	-1.3 ± 0.1	-1.6 ± 0.5
B yield (g/g)	0.09 ± 0.00[a]	0.11 ± 0.02[a]
IBE yield (g/g)	0.18 ± 0.02[a]	0.16 ± 0.03[a]
IBE productivity (g/L·h)	0.14 ± 0.01[a]	0.14 ± 0.00[a]

TRS: total reducing sugar.

Means and standard deviations followed by different letters are statistically different from each other (Tukey's test at 95% confidence interval).

[1]Negative concentration means that the final concentration was lower than the initial concentration.

As for the defined media, the lesser was the amount of sucrose in the medium, the higher was the IBE yield (Figure 2.3.3). Moreover, the utilization of sucrose was in general lower compared with glucose, fructose, and xylose. For example, in the fermentation medium S + G + F + X (70 g/L initial TRS), the following values of conversion were measured: 21% (sucrose), 52% (glucose), 46% (fructose), and 51% (xylose). When this fermentation was repeated with a lesser amount of sucrose (40 g/L initial TRS), the conversion of sucrose increased: 42% (sucrose), 52% (glucose), 63% (fructose), and 52% (xylose). Nevertheless, the mass fraction of sucrose in the remaining sugar (at the end of the fermentation) increased regardless of the initial sugar

concentration (Figure 2.3.3). Overall, the incomplete utilization of sucrose, fructose, and xylose is a problem that has yet to be solved for more efficient utilization of mixed hydrolysates – supplemented with molasses – for IBE production.

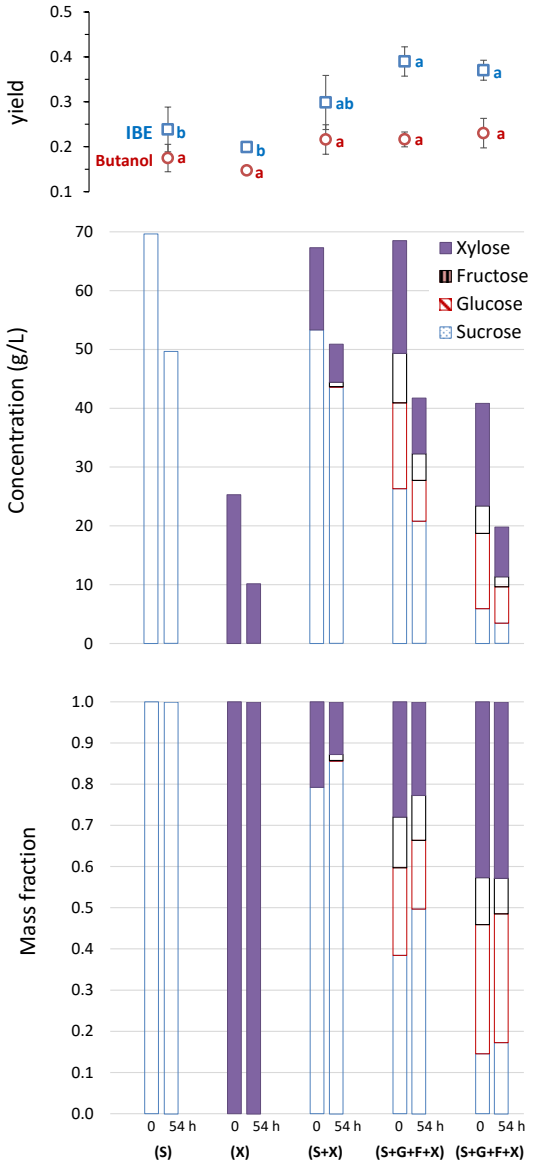


Figure 2.3.3. IBE and butanol yields from laboratory-grade sugars in batch culture of *C. beijerinckii* DSM 6423, and respective sugar concentration and sugar mass fraction at initial and final fermentation time. Means of yield followed by different letters are statistically different from each other (Tukey's test at 95% confidence interval). S: sucrose; X: xylose; G: glucose; F: fructose; X: xylose.

2.3.4. Conclusions

C. beijerinckii DSM 6423 can consume lactic acid and this characteristic improved the butanol yield from bagasse hydrolysates. Moreover, molasses can be an efficient source of nutrients and buffering capacity. Remarkably, *C. beijerinckii* DSM 6423 can co-ferment sucrose, glucose, fructose, and xylose while consuming lactic acid. However, more efficient utilization of sucrose and xylose is still a challenge. Finally, given the ability of *C. beijerinckii* DSM 6423 to consume lactic acid, the enzymatic hydrolysis in future IBE plants may be operated under non-sterile conditions, counterbalancing sugar losses and saving costs related to microbial contamination control.

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3. FUTURE PUBLICATION

3.1. Using 3D-printing to build the immobilization system of repeated-batch fermentation integrated with vacuum extraction for isopropanol-butanol-ethanol production

This section comprises the study of vacuum extractive fermentation applied to immobilizing IBE fermentation by *Clostridium beijerinckii* DSM 6423. The immobilization system is composed by the sugarcane bagasse trapped in a cage-like polymeric structure produced in a 3D printer. The evaluation of the 3D cage-like structure is presented in Appendix B. This section achieves objectives 2 and 3 of section 1.2. This is the first draft of the paper related to this section (*Paper 4*).

We would like to acknowledge FAPESP (grant number 2016/23042-9) for providing financial resources for the participation in the 12th European Congress of Chemical Engineering occurred in Florence, Italy (2019). The discussions about the bioreactor setup occurred during this event were very important for concluding this chapter. We would like to offer a special acknowledgement for Dra. Eloísa Rochón for kindly offer her knowledge to help in this issue.

ABSTRACT

Butanol toxicity is known to limit its production through fermentation process, and IBE producer bacteria are especially sensitive to this solvent; their metabolism are inhibited by butanol concentrations as low as 5 g/L. Thus, cell immobilization technology using sugarcane bagasse trapped in a 3D prototype that avoided clogging was used as cell carrier in repeated-batch IBE fermentation. Compared to conventional single-batch fermentation, 200 % increase in IBE production was reported for the immobilized repeated-batch process. However, despite final titers gains, substrate losses were still observed due to butanol toxic effects (almost 60 % sugar loss). Integrating online solvent removal by vacuum at the immobilized repeated-batch fermentation allowed sugar exhaustion in one of the batch cycles, increased overall sugar consumption (66 % sugar consumption), and generated a concentrated product stream (49.2 g/L IBE, 29 g/L butanol), offering economic opportunities to decrease energy demands towards distillation.

Keywords: Butanol, sugarcane bagasse, repeated-batch, vacuum fermentation, *Clostridium beijerinckii,* 3D-printing.

3.1.1. Introduction

Biobutanol has gained increasing attention upon the biofuel market due to its "drop in" characteristics and higher energy density compared with ethanol. The global biobutanol market, valued at US$ 90 million in 2020, is expected to achieve US$ 114.7 million by the end of 2026 (Market Watch, 2020). However, its current production based on ABE (acetone-butanol-ethanol) fermentation is challenged by the presence of acetone, which is corrosive and has poor fuel properties (Li et al., 2019). Considering the scenario where biobutanol production achieves billions of liters due to biofuels demands, an oversupply of acetone can be expected. In response to this challenge, researchers have been investing in the IBE (isopropanol-butanol-ethanol) fermentation, where only fuel alcohols are produced (Vieira et al., 2019).

The IBE fermentation is performed mainly by clostridia bacteria capable of reducing acetone into isopropanol through a primary-secondary alcohol dehydrogenase enzyme (Vieira et al., 2019). This ability to produce only fuel alcohols is the main advantage over ABE fermentation. Indeed, the IBE mixture can be used directly as fuel or as gasoline additive in any proportion, decreasing biobutanol production market risks related to the presence of acetone (Li et al., 2019). Although, technical limitations, extensively studied for the ABE fermentation (Otraum et al., 2017; Koleinska et al., 2019), are even more accentuated in the IBE fermentation (Vieira et al., 2019).

Low cell density and, consequently, low fermentation productivity, for example, are important drawbacks of the biobutanol production process. In free-cell single-batch cultivations, while ABE fermentation normally achieves 0.30 g ABE/L.h average productivity and 16-17 g/L average final butanol titer (Koleinska et al., 2019), our recently published review showed that IBE fermentation productivity is limited to 0.15 g IBE/L·h average and final butanol titer does not exceed 10 g/L (Vieira et al., 2019).

To overcome this limitation, researchers have been applying cell immobilization technology with lignocellulose material as cell carrier, for both ABE (Qureshi et al., 2015; Abo et al., 2019) and IBE (Vieira et al., 2019) fermentation processes.

Besides the increase of cell density and fermentation productivity, the use of a cell carrier also allows the reutilization of the grown cells in repeated-batch processes, eliminating the necessity of inoculum preparation and reducing lag-phases, which also contribute to productivity gains (Koleinska et al., 2019). However, the long-term stability of the immobilized cells used in repeated-batch cultivations can be compromised by butanol toxicity, another common drawback of both ABE and IBE fermentation, which causes incomplete sugar conversion, productivity losses and eventual cell degeneration (Qureshi et al., 2005).

In fact, we recently published a study where sugarcane bagasse was used as cell carrier for IBE production through repeated-batch cultivations and decreases in IBE yields and final butanol titers and productivities over the batch cycles were reported. Nevertheless, in four of the batch cycles of the best fermentation experiment conditions, IBE productivity was between 0.22 and 0.28 g/L·h, and butanol titer achieved 6.7–8.6 g/L. However, overall sugar conversion was approximately 68 %, which caused almost 135 g/L glucose loss (Vieira et al., 2020). Therefore, we could hypothesize that the gains promoted by cell immobilization using sugarcane bagasse as cell carrier for IBE fermentation could be improved if butanol toxicity effects were diminished by recovery techniques (Kujawska et al., 2015; Otraum et al., 2017).

Actually, cell immobilization and butanol recovery have been used together in biobutanol production through ABE fermentation, and significant gains have been reported (Liu et al., 2014; Cai et al., 2015; Cai et al., 2016; Li et al., 2016). Considering this scenario of integrated technologies, vacuum fermentation appears to be especially

compatible with cell immobilization using lignocellulose material as cell carrier. During vacuum fermentation, the pressure inside the bioreactor is reduced by a vacuum pump, which causes solvents vaporization at the fermentation temperature; then, solvents are captured by a condensation system. This technology does not comprise membranes or gas stripping. Therefore, it is not susceptible to clogging by lignocellulose fibers. Besides, vacuum extraction offers high butanol removal rates (between 1.4 and 16.6 g/L·h), approximately 10 times "faster" than gas stripping (Mariano et al., 2011), which makes this technology suitable for high cell concentration systems offered by immobilized fermentation. However, this technology has never been applied in immobilized systems for IBE fermentation.

Thus, here we proposed a fermentation configuration where we coupled vacuum extraction technology and an immobilization system using sugarcane bagasse as cell carrier at the same vessel for IBE production through repeated-batch fermentation. To avoid clogging problems by sugarcane bagasse, a spiral-wound (Kilonzo et al., 2010) 3D printed cage-like polymeric prototype was used to trap the sugarcane bagasse inside the bioreactor. This immobilization system presents as an advanced technology since 3D printing was used to develop the fermentation configuration.

3.1.2. Material and Methods

3.1.2.1. Microorganism and inoculum preparation

Clostridium beijerinckii DSM 6423 performed IBE fermentation. Bacteria spores were stored in sterile distilled water (spore solution) under 4 °C. For inoculum preparation, 3.2 mL spore solution were heated at 75 °C for 10 min, followed by 2 min ice cooling. This aliquot was transferred to 80 mL anoxic pre-sterilized TGY medium and incubated in anaerobic chamber (COY Type A vinyl chamber) at 35 °C for 18 hours. Finally, the 80 mL grown cells were transferred to 720 mL TGY medium and

incubated for more 5 hours under the same conditions (fermentation inoculum). TGY medium composition: g/L, 30 tryptone, 20 glucose, 10 yeast extract, 1 L-cysteine.

3.1.2.2. Design and printing of the 3D polymeric prototype

The cage-like 3D polymeric prototype used to trap the sugarcane bagasse consisted of four concentric cylinders of 12.0 mm thickness, interspaced by 10.0 mm; the prototype height and diameter were 80.0 and 162.2 mm, respectively; the orifice diameter (which allowed the contact between the sugarcane bagasse and the fermentation medium) was 2.0 mm. The software Solid Works was used for designing (Figure 3.1.1). The prototype was printed at the National Institute of Biofabrication (BIOFABRIS) using a rapid prototyping 3D printer (laser sintering) EOS FORMIGA P110 and nylon PA 2200 as material.

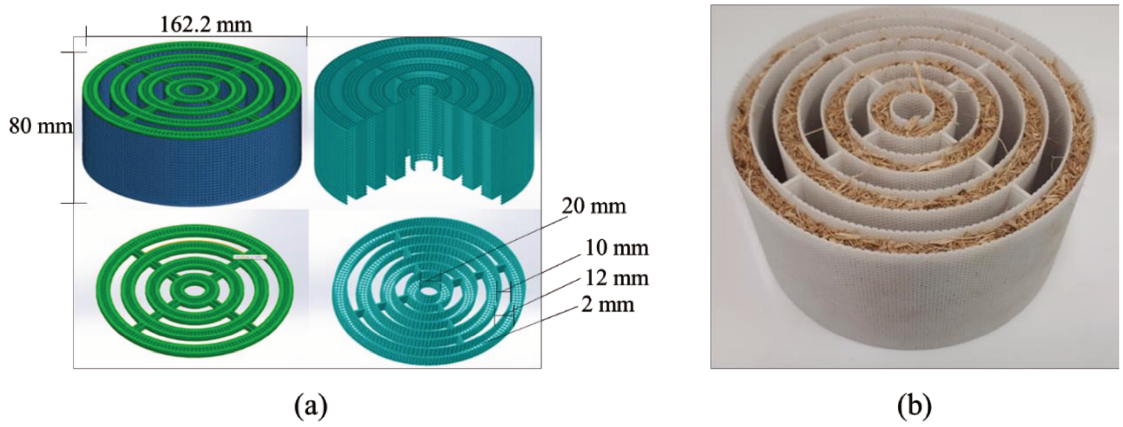

Figure 3.1.1 – 3D prototype design on Solid Works (a); and sugarcane bagasse trapped inside the prototype (b).

3.1.2.3. Free-cell single-batch fermentation

Free-cell single-batch fermentation was conducted in a 5-L bioreactor (Bioflo & Celligen 310, New Brunswick) containing 4 L 60 g/L glucose P2 medium (Vieira et al., 2020). The bioreactor containing the fermentation medium (glucose and yeast extract solution) was autoclaved at 121 °C for 20 min followed by cooling to 35 °C under O_2-free N_2 atmosphere. Once the bioreactor temperature had stabilized, filter-sterilized

(0.22 μm) P2 stock solutions (buffer, mineral, and vitamin solutions) and 20 % inoculum were added. O_2-free N_2 gas was flushed in the bioreactor headspace during the first 18 hours of fermentation; during this period, the bacteria had time to produce enough fermentation gases (CO_2 and H_2) to keep the bioreactor anaerobic. Fermentation temperature and agitation were 35 °C and 50 rpm, respectively.

3.1.2.4. Immobilized repeated-batch fermentation using sugarcane bagasse as cell carrier

Sugarcane bagasse as cell carrier for *Clostridium beijerinckii* DSM 6423 (Vieira et al. 2020) was trapped in the cage-like 3D polymeric prototype (section 3.1.2.2) and placed inside the bioreactor. Further procedures for bioreactor preparation including the fermentation medium, temperature, and agitation were described in section 3.1.2.3. Three different experiment conditions were tested varying sugarcane bagasse to liquid ratio and time of each batch cycle (Table 3.1.1).

Table 3.1.1 – Variations in the repeated-batch fermentation experiment without vacuum extraction.

	Experimental Condition		
	I	II	III
Bagasse to liquid ratio	1:75	1:40	1:40
Time of each batch cycle (h)	18-18-23	12-12-21-36-68	18-24-24-24-48

3.1.2.5. Immobilized single- and repeated-batch fermentation with vacuum extraction using sugarcane bagasse as cell carrier

Immobilized fermentation experiments with vacuum extraction were conducted with free and trapped sugarcane bagasse as cell carrier. Free-bagasse fermentation was conducted in single-batch mode due to operational difficulties to perform a repeated-batch process. Trapped-bagasse fermentation was conducted in repeated-batch mode totalizing five batch cycles. The fermentation system was composed by the bioreactor (sections 3.1.2.3 and 3.1.2.4), a condenser, a circulating bath (Chiller Marconi MA184), a vacuum pump (Fisatom 826), and an exhaust trap (1 L screw capped bottle containing 500 mL distilled water) (Figure 3.1.2).

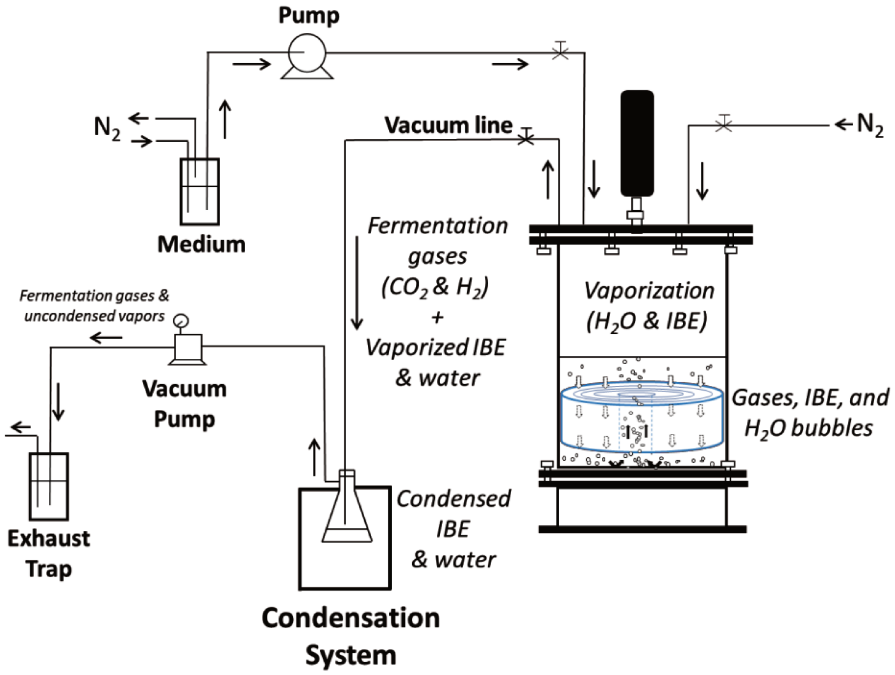

Figure 3.1.2 – Immobilized fermentation with vacuum extraction system.

The vacuum pump decreased the pressure inside the bioreactor (8 kPa), and consequently, the media boiled at the fermentation temperature (35 °C) generating vapors (IBE + water) that were condensed in a glass coil condenser placed between the bioreactor and the vacuum pump. Ethyleneglycol-water solution (50% v/v) cooled at -4 °C was circulated around the condenser. Vacuum pump exhaust (fermentation gases and uncondensed vapors) was bubbled in the exhaust trap to collect escaping IBE vapors. After vacuum sessions, samples were taken by restoring the pressure with O_2-free N_2 gas. Antifoam 204 Sigma was used when needed. Sugarcane bagasse to liquid ratio was 1:40 for both free- and trapped-bagasse experiments. Intermittently 2h-vacuum sessions were separated by varied intervals according Figure 3.1.3.

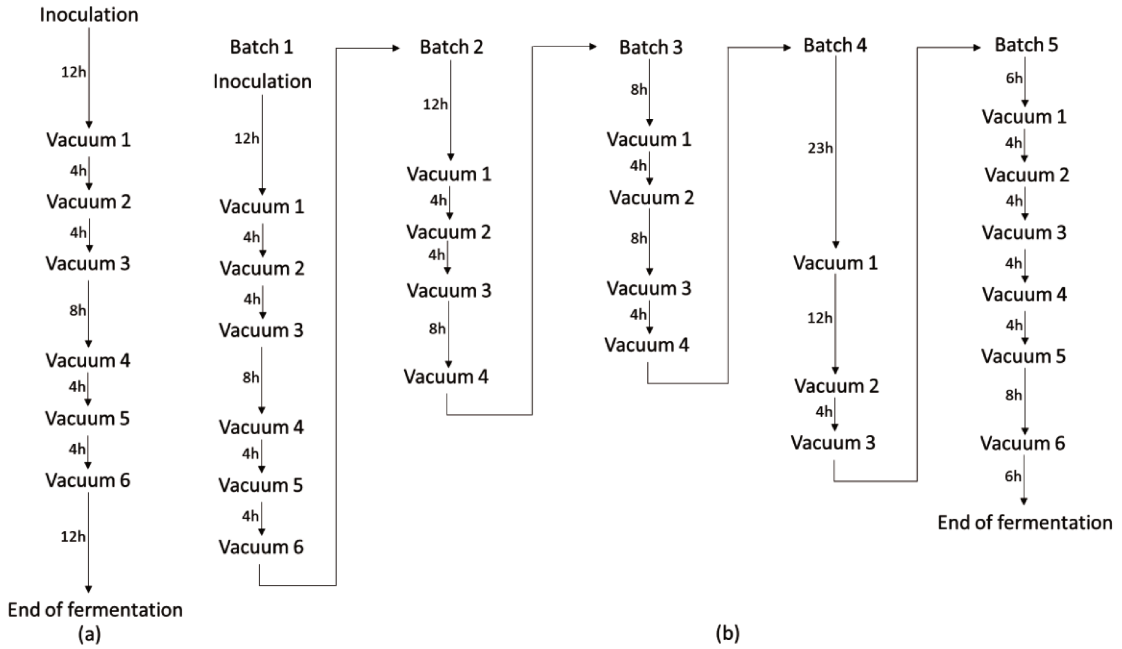

Figure 3.1.3 – Scheme of intermittent 2 hours vacuum sessions. Free bagasse single-batch fermentation (a); and trapped bagasse repeated-batch fermentation (b).

3.1.2.6. Analytical methods and calculations

Glucose, acetic acid, butyric acid and IBE were separated and measured by high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity) using a Bio-Rad Aminex® HPX-87H column (15 °C temperature, 3 mM H_2SO_4 mobile phase, 0.5 mL/min flow rate); refractive index detector (RID) was used. IBE productivity (g/L·h) was calculated as amount of IBE produced (g/L) divided by fermentation time (h). IBE yield (g/g) was defined as the amount of IBE produced (g/L) per total consumed glucose (g/L). Glucose conversion (%) was calculated as amount of glucose consumed (g/L) divided by the amount of initial glucose (g/L). In immobilized fermentation experiments with vacuum extraction, the amount of product produced (g) consists of products collected in the condenser and the exhaust trap added by the amount of product remained in the reactor at the end of the fermentation subtracted by the amount of product inside the reactor at the beginning of the fermentation (from inoculum). Product concentration in the condensate (g/L) was calculated as the amount of product collected

in the condenser and the exhaust trap (g) divided by the condensate volume (L). Product removal rate (g/L·h) is defined as the amount of product in the condensate (g) divided by the fermentation volume (4L) and total time of vacuum sessions (h).

3.1.3. Results and Discussion

3.1.3.1. Single-batch fermentation

Clostridium beijerinckii DSM 6423 was severely affected by the presence of butanol (Figure 3.1.4).

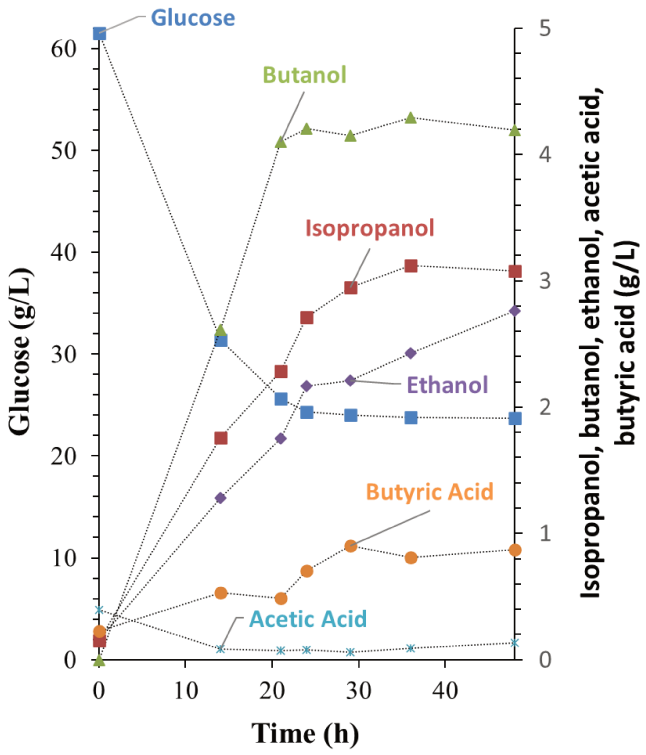

Figure 3.1.4 – Single-batch fermentation results.

In approximately 36 hours of fermentation, butanol concentration reached 4.3 g/L, which apparently inhibited *C. beijerinckii*. At this stage of fermentation, isopropanol, ethanol, acetic acid, and butyric acid concentrations were 3.1, 2.4, 0.09, and 0.72 g/L respectively; glucose conversion was 61.3 %, with remaining 23.8 g/L glucose, which represents almost 40 % substrate loss. IBE yield and productivity were 0.26 g/g and 0.27 g/L·h, respectively. After that, no significant amount of IBE was produced.

Previous results of IBE fermentation by *Clostridium beijerinckii* DSM 6423 show that butanol concentrations between 3.7 (Survase et al., 2011) and 5 g/L (Vieira et al., 2020) inhibited the bacteria, causing substrate losses (other IBE single-batch fermentation results can be found in Vieira et al., 2019). On the other hand, when ABE single-batch fermentation was performed by *Clostridium beijerinckii* P260 inside a bioreactor also using glucose P2 medium, butanol concentration reached 11.8 g/L with 23.6 % substrate loss (Mariano et al., 2011). These results confirm that IBE fermentation is more sensitive to butanol toxicity than ABE fermentation, which makes online product recovery techniques even more necessary to guarantee higher substrate conversion and solvents productivity.

3.1.3.2. Immobilized single-batch fermentation with vacuum extraction using free sugarcane bagasse as cell carrier

Due to bioreactor draining tubes clogging by sugarcane bagasse, free-bagasse immobilized fermentation with vacuum extraction was conducted in only single-batch mode, since we were not able to drain the bioreactor at the end of the batch cycle. This operational problem also prevented samples withdrawal without opening the bioreactor, leading to O_2 contamination along the process. Nevertheless, fermentation results showed that vacuum extraction did not allow butanol and IBE levels to exceed 2.0 and 3.0 g/L, respectively. However, sugar consumption was not complete (almost 61 % glucose consumption), even after 60 hours of fermentation, probably because of the O_2 contamination. OD achieved high levels; although, this analysis was compromised by the presence of sugarcane bagasse small fragments, that elevated the medium turbidity even after sample centrifugation (Figure 3.1.5).

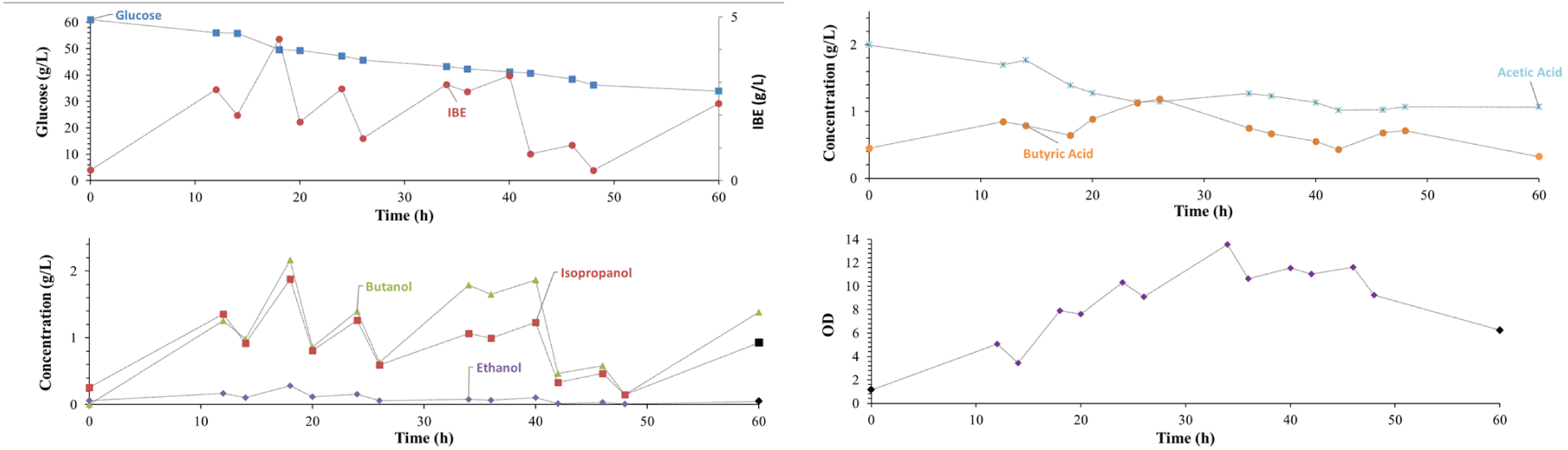

Figure 3.1.5 – IBE production through single-batch fermentation with vacuum extraction using free sugarcane bagasse as cell carrier for *Clostridium beijerinckii*.

Nevertheless, a condensate with 14.1 g/L butanol and 23.3 g/L IBE was obtained at the end of the fermentation. This more concentrated product stream can decrease distillation costs, increasing economic viability of IBE production (Mariano et al., 2011). However, IBE yield and productivity were 0.22 g/g and 0.14 g/L·h, lower than those of the conventional single-batch fermentation (Table 3.1.3).

3.1.3.3. Immobilized repeated-batch fermentation using sugarcane bagasse trapped inside the 3D prototype as cell carrier

To avoid operational problems regarding clogging, further experiments were conducted using a spiral-wound cage-like 3D-printed prototype to trap the sugarcane bagasse inside the bioreactor (Kilonzo et al., 2010).

Glucose conversion, IBE productivity, OD increase, and final butanol and IBE concentrations of the first batch of experiment I were the highest among all batch cycles reported in Table 3.1.2. However, despite the high amount off cells grew during the first batch (OD increased from 0.11 to 3.38), sugar conversion, and IBE production and productivity were lower for consecutive batches. This probably occurred due to cell exposure to high concentrations of butanol (Qureshi et al., 2015). For this reason, in experiment II, we reduced the time of the first batch from 18 to 12 h, and increased bagasse loading from 1:75 to 1:40 [in Vieira et al., 2020, we showed that higher sugarcane bagasse amounts can benefit the fermentation, possibly due to increased biofilm formation and its protection barrier against butanol (Qureshi et al., 2015)].

Although the change allowed the conduction of five consecutive batches, the first batch was characterized by low glucose conversion and IBE production. Nevertheless, the lower exposure to butanol and the higher amount of sugarcane bagasse allowed the cells to perform a better second batch, with better results regarding sugar conversion and IBE production, and the maintenance of IBE yield. Remarkably, during the second

batch, OD increased from 0.47 to 3.33, indicating high cell growth. However, consecutive batches showed almost nonsolvent production and consequent IBE yield decline. Concentrations of organic acids were higher, reaching 5.98 g/L butyric acid at the end of the 5th batch. Such results could indicate an "acid crash" (term normally used in the industry when excess of acid is produced by solventogenic microorganisms) (Maddox et al., 2000).

Table 3.1.2 – Repeated-batch fermentations for IBE production.

Experimental Conditions	Parameter	Repeated Batches				
		B1	B2	B3	B4	B5
I	Initial glucose (g/L)	62.3	61.0	70.7	-	-
	Glucose conversion (%)	69.1	18.5	44.4	-	-
	Fermentation time (h)	18	18	23	-	-
	Initial OD (540 nm)	0.11	1.74	1.12	-	-
	Final OD (540 nm)	3.38	1.64	1.45	-	-
	I (g/L)	3.4	0.4	1.6	-	-
	B (g/L)	6.8	2.7	2.5	-	-
	E (g/L)	0.5	0.0	0.3	-	-
	IBE (g/L)	10.7	3.1	4.4	-	-
	Acetic acid (g/L)	-0.03	-0.22	-1.36	-	-
	Butyric acid (g/L)	0.16	0.43	2.38	-	-
	I:B:E mass ratio	6.8:12.8:1	-	5.3:8.2:1	-	-
	IBE yield (g/g)	0.25	0.27	0.14	-	-
	IBE productivity (g/L·h)	0.59	0.17	0.19	-	-
II	Initial glucose (g/L)	59.8	52.6	52.0	52.6	49.2
	Glucose conversion (%)	16.8	40.8	22.5	33.6	56.3
	Fermentation time (h)	12	12	21	36	68
	Initial OD (540 nm)	0.09	0.47	3.34	0.60	1.41
	Final OD (540 nm)	1.31	3.33	1.52	3.50	2.98
	I (g/L)	0.6	1.9	0.3	0.0	0.0
	B (g/L)	1.9	3.4	0.5	0.9	3.2
	E (g/L)	0.1	0.2	0.1	0.0	0.1
	IBE (g/L)	2.7	5.5	0.9	0.9	3.4
	Acetic acid (g/L)	0.14	-0.31	1.42	0.84	0.85
	Butyric acid (g/L)	1.15	2.25	4.09	5.20	5.96
	I:B:E mass ratio	6:19:1	9.5:17:1	3:5:1	-	0:32:1
	IBE yield (g/g)	0.26	0.26	0.08	0.06	0.12
	IBE productivity (g/L·h)	0.22	0.46	0.04	0.03	0.05
III	Initial glucose (g/L)	56.2	48.1	46.0	44.7	48.5
	Glucose conversion (%)	39.0	37.4	53.3	35.7	19.3
	Fermentation time (h)	18	24	24	24	48
	Initial OD (540 nm)	0.22	2.36	1.61	0.99	0.80
	Final OD (540 nm)	1.54	3.10	2.33	1.76	0.64
	I (g/L)	2.4	2.4	3.4	1.6	0.8
	B (g/L)	4.4	3.6	4.9	2.6	1.4
	E (g/L)	0.4	0.4	0.5	0.2	0.1
	IBE (g/L)	7.3	6.4	8.8	4.5	2.3
	Acetic acid (g/L)	-0.65	0.04	-0.91	-0.13	0.76
	Butyric acid (g/L)	0.44	0.17	0.13	1.91	1.00
	I:B:E mass ratio	6:11:1	6:9:1	6.8:9.8:1	8:13:1	8:14:1
	IBE yield (g/g)	0.33	0.36	0.36	0.28	0.25
	IBE productivity (g/L·h)	0.41	0.27	0.37	0.19	0.05

Probably, experiment II conditions stimulated high and fast cell growth during the second batch. The growth phase of solventogenic Clostridia is characterized by acetic and butyric acids production. However, apparently, *C. beijerinckii* DSM 6423 was not

able to reassimilate the acids and produce IBE, which caused the “acid crash” (Maddox et al., 2000). We believe the 12 hours of the first batch were not enough for the bacteria to grow and produce biofilm, which could both prevent excessive growth during the second batch and improve their tolerance against butanol toxicity (Survase et al. 2013; Yang et al. 2016; Zhang et al. 2016).

Therefore, for experiment III we decided to maintain bagasse loading at 1:40 and increase the fermentation time of the first batch from 12 to 18 hours. Despite the second batch lower IBE production and productivity, this strategy allowed the maintenance of IBE concentrations and yields above 6.4 g/L and 0.33 g/g, respectively, during the first three batches (66 hours of fermentation process). However, consecutive batches showed decreasing sugar conversions, final IBE concentrations, yields and productivities (Table 3.1.2), probably caused by cell exposure to butanol concentrations above 6.0 g/L during the first three batches.

In comparison with the single-batch fermentation, repeated-batch experiments I and II had lower average IBE yields, and experiments II and III had lower average IBE productivities (Table 3.1.3). Although, the lower productivities could be explained by the longer fermentation time (149 and 138 hours, respectively, against 36 hours for the single-batch fermentation). Moreover, glucose conversion was lower for all conditions (Table 3.1.3). These results show that the immobilization system was not enough to improve fermentation performances of all repeated-batch experiments, which were severely affected by butanol toxicity.

3.1.3.4. Immobilized repeated-batch fermentation with vacuum extraction using sugarcane bagasse trapped inside the 3D prototype as cell carrier

At the beginning of the fermentation process we had operational problems related to excessive foam. Therefore, the first three vacuum sessions of the first batch cycle

were not adequate for keeping butanol and IBE concentrations below toxic levels; between 18 and 34 hours of fermentation, butanol and IBE concentrations remained above 5 and 9 g/L, respectively. Only the fourth vacuum session was effective; butanol and IBE concentrations decreased from 5.2 and 12.6 g/L to 2.1 and 3.6 g/L, respectively. Consequently, glucose exhaustion was not achieved at the end of the first batch, probably due to butanol toxicity effects (Figure 3.1.6).

Nevertheless, vacuum extraction allowed the conduction of 48 hours of the first batch cycle, higher than 12 or 18 hours performed in the repeated-batch experiments without vacuum extraction. Due to this longer batch period, the bacteria cells had time to grow and produce biofilm. Consequently, for the second batch, the already existing amount of cells and the vacuum extraction (butanol and IBE concentrations were below 3.5 and 6 g/L, respectively, during the 36 hours of fermentation) allowed almost complete glucose conversion (5.6 g/L glucose remained). Besides, OD achieved its higher value, indicating elevated cell growth (Figure 3.1.6).

However, for the third batch cycle, IBE production was low, indicating low sugar consumption. Indeed, glucose concentration increased due to liquid evaporation (which reduces liquid volume inside the reactor, increasing non-volatile components concentration) caused by vacuum extraction. Again, as we reported for repeated-batch experiments without vacuum extraction, high concentrations of acetic and butyric acids were observed, indicating a possible “acid crash”, probably due to excessive cell growth during the second batch cycle (Figure 3.1.6c) (Maddox et al., 2000).

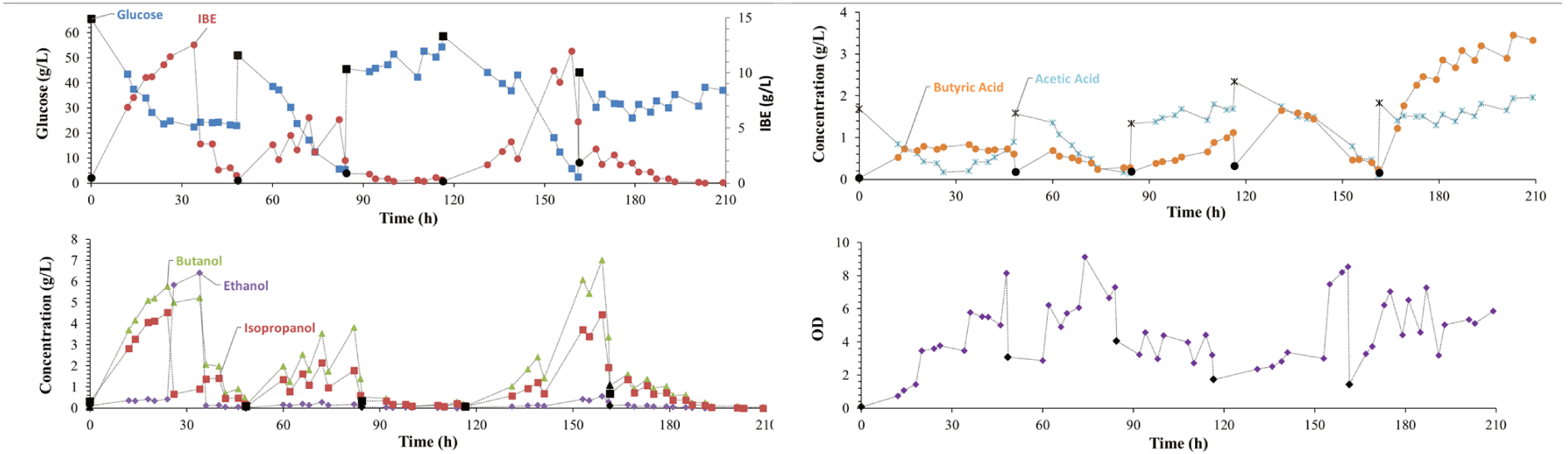

Figure 6 – IBE production through repeated-batch fermentation with vacuum extraction using trapped sugarcane bagasse as cell carrier for *Clostridium beijerinckii*. Black symbols mean beginning of a batch cycle.

Nevertheless, during the fourth batch, immobilized *Clostridium beijerinckii* was able to consume all glucose available after 45 hours, and butanol concentration achieved its highest value (7.0 g/L), indicating high butanol production during this batch cycle. However, excessive cell growth was observed again (OD achieved 8.5). Consequently, the fifth batch cycle was characterized by high acetic and butyric acids production, and low glucose consumption and solvent production (Figure 3.1.6).

Regardless, the gains offered by vacuum extraction are important. Glucose conversion, total IBE production and IBE yield increased significantly compared with all immobilized repeated-batch fermentation experiments without vacuum extraction. *Clostridium beijerinckii* DSM 6423 was able to perform 209 hours of fermentation, which is also higher than the fermentation time reported for experiments I, II, and III; 59, 149 and 138 h, respectively (Table 3.1.3).

Table 3.1.3 – Final fermentation results for IBE production through conventional single-batch fermentation, immobilized repeated-batch fermentation, and immobilized repeated-batch fermentation with vacuum extraction.

Parameter	Conventional single-batch fermentation	Immobilized repeated-batch fermentation			Immobilized repeated-batch fermentation with vacuum extraction using free sugarcane bagasse	Immobilized repeated-batch fermentation with vacuum extraction using trapped sugarcane bagasse
		I	II	III		
Glucose conversion (%)	61.3	44.2	33.3	36.9	61.6	65.7
Fermentation time (h)	36	59	149	138	60	209
Total isopropanol produced (g)	12.5	21.5	11.4	42.8	11.9	82.4
Total butanol produced (g)	17.2	47.7	39.8	68.0	21.3	136.9
Total ethanol produced (g)	9.7	2.9	2.5	6.6	0.8	11.9
Total IBE produced (g)	39.4	72.1	53.6	117.4	35.4	231.2
I:B:E mass ratio	1.3:1.8:1	7.5:16.6:1	4.6:16.2:1	6.5:10.3:1	14.9:26.6:1	7:11.6:1
Total acetic acid produced (g)	-1.21	-6.50	11.80	-3.60	-5.03	-7.02
Total butyric acid produced (g)	2.58	11.0	74.6	14.6	-0.12	20.74
IBE yield (g/g)	0.26	0.21	0.15	0.33	0.23	0.35
IBE productivity (g/L·h)	0.27	0.31	0.09	0.21	0.14	0.28
Isopropanol in condensate (g/L)	-	-	-	-	8.4	18.1
Butanol in condensate (g/L)	-	-	-	-	14.1	29.0
Ethanol in condensate (g/L)	-	-	-	-	0.7	2.2
IBE in condensate (g/L)	-	-	-	-	23.3	49.2
Acetic acid in condensate (g/L)	-	-	-	-	0.00	0.69
Butyric acid in condensate (g/L)	-	-	-	-	0.64	1.80
Condensate volume (L)	-	-	-	-	1.24	4.22
Broth evaporation (% of broth volume)	-	-	-	-	31.0	22.4
Total vacuum time (h)	-	-	-	-	12	46
IBE removal rate (g/L·h)	-	-	-	-	1.94	1.07

Although IBE productivity was lower compared to experiment I, this could be justified by the higher fermentation time (209 against 59 hours). Considering experiment III (obtained better fermentation results among all repeated-batch experiments without vacuum extraction), the addition of vacuum extraction technology offered a productivity gain of 33 % (Table 3.1.3). This would decrease the number of fermentation tanks required by a 100-kt/a IBE plant from 15 to 11 (3785 m^3 each unit) (Vieira et al., 2019). However, further studies are needed to adjust fermentation conditions concerning vacuum extraction, such as time for beginning the sessions and duration of each session, to avoid exposure of the cells to high concentrations of butanol, which happened during the first and the fourth batch. Moreover, continuous fermentation process could be studied to avoid cell stress due abrupt sugar concentration increase, normally occurred during a repeated-batch fermentation process. For example, if the third and the fifth batches (which were severely affected by cell stress due to butanol toxicity and abrupt sugar concentration increase) were excluded from the process, the fermentation productivity would be 0.35 g/L·h. This represents productivity gain of 25 % compared to the whole process, which would decrease to 9 the number of fermentation tanks.

Finally, a 49.2 g/L IBE condensate with 29.0 g/L butanol was obtained from the trapped-bagasse immobilized repeated-batch fermentation with vacuum extraction (Table 3.1.3). In contrast, the highest final IBE and butanol concentration remained inside the bioreactor in this research after an immobilized single-batch fermentation was 10.7 and 6.8 g/L, respectively (Table 3.1.2). This represents a product stream almost 5 times more IBE concentrated, and 4 times more butanol concentrated. Since reduced distillation energy demands are required for more concentrated product streams, we can expect economic gains in downstream processes (Mariano et al., 2011).

3.1.4. Conclusions

IBE fermentation is limited by excessive bacteria sensitivity towards butanol. Cell immobilization technology using sugarcane bagasse as cell carrier allowed higher titers, and the possibility of reusing grown immobilized cells in repeated-batch processes. However, sugar conversion and fermentation time were still limited by butanol toxicity. On-line solvent removal by vacuum increased final butanol production, allowed longer fermentation time, and reduced substrate loss through a more complete sugar conversion. Besides, the production of a solvent concentrated condensate at the end of the process can offer economic gains due to lower distillation energy requirements.

Acknowledgements

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4. DISCUSSIONS

Nowadays, biobutanol is produced through ABE fermentation, where acetone, butanol and ethanol are produced. However, acetone, that represent 20-30% of the ABE mixture, is corrosive and has poor fuel properties. For this reason, researchers have been investigating the IBE mixture, where only fuel alcohols (isopropanol, butanol and ethanol) are produced. However, technical problems including low cell density, low butanol titers, low productivities, high product inhibition and incomplete sugar conversion are present in the fermentation process. Moreover, current environment scenario requires the use of renewable biomass, such as sugarcane bagasse lignocellulose material, as source of biofuels.

Thus, this thesis proposed the development of an innovative fermentation technology for IBE production combining cell immobilization technology in sugarcane bagasse trapped in a 3D-printed cage-like polymeric structure, and in-situ product recovery by vacuum, in the same fermentation tank. In parallel, sugarcane bagasse second-generation feedstock was evaluated as substrate for IBE fermentation.

Our first published results showed the use of sugarcane bagasse as cell carrier for the IBE producer *Clostridium beijerinckii* DSM 6423 was effective in improving fermentation IBE productivities and final IBE titers. This may have occurred due to higher cell density inside the bioreactor stimulated by the presence of the cell carrier. Besides, in repeated-batch experiments, our results also showed improvements in sugar conversion, and increased IBE productivities and final IBE titers when compared to single-batch cultivations.

The used of cell immobilization techniques using sugarcane bagasse as cell carrier in the 7-liters bioreactor required a structure to trap the material inside the bioreactor during the fermentation process to avoid bagasse flotation and clogging. Therefore, a 3D-printed prototype was used for this purpose. This immobilization system was coupled with the vacuum extraction system attempting to improve fermentation performance by increasing cell density and reducing product inhibition. Experiments results showed benefits in the fermentation process. A 209h-

repeated-batch IBE fermentation was conducted and a condensate with approximately 50 g/L IBE (g/L, isopropanol 18.1, butanol 29.0, ethanol 2.2) was obtained. Besides improving fermentation parameters such as final IBE titers and productivities, and sugar consumption, the recovery of an IBE concentrated stream can benefit downstream processes.

Finally, we tested second-generation substrates to produce IBE. Sugarcane bagasse cellulose and hemicellulose hydrolysates were tested and, despite the presence of lactic acid from microbial contamination in the cellulose hydrolysate, *C. beijerinckii* was able to consume the lactic acid and the glucose to produce IBE. When the hemicellulose hydrolysate was used as fermentation medium the IBE fermentation did not occur. However, mixing the cellulose hydrolysate with the hemicellulose hydrolysate allowed the IBE production. Moreover, when molasses was used as nutrient and buffer supplementation, fermentation parameters improved, show molasses can be a good alternative to laboratory grade supplementation.

5. CONCLUSIONS

Firstly, the sugarcane bagasse was evaluated as cell holding material for *C. beijerinckii* DSM 6423 to perform the IBE fermentation. Experiments showed that glucose conversion, IBE productivity and final butanol titer in free-cell single-batch cultivation were limited to 35 %, 0.13 g IBE/L·h, and 4.8 g/L, respectively. The addition of sugarcane bagasse as cell carrier improved fermentation parameters; glucose conversion, IBE productivity and final butanol titer were 72 %, 0.24 g/L·h, and 7.1 g/L, respectively. In a long-term process, *C. beijerinckii* was able to perform seven consecutive batches (257 h), in which glucose conversion varied between 38 % and 98 %; in four of these consecutive batches, IBE productivity and final butanol titer achieved values between 0.22 and 0.28 g/L·h, and 6.7 e 8.6 g/L, respectively.

Then, the 3D printed cage-like prototype geometry used to trap the sugarcane bagasse in the bioreactor was evaluated. The most promising geometry concerning mass transfer consisted of four perforated (which allowed the contact of the sugarcane bagasse with the medium) concentric cylinders, separated by empty spaces, which allowed the motion of the bubbles generated during vacuum evaporation. This immobilization system was used in repeated-batch IBE fermentations with and without *in-situ* product recovery by vacuum.

In the experiments where the vacuum technology was not used, we were able to conduct five consecutive batches, which lasted 138 hours; glucose conversion was limited to 37 %, and IBE productivity was 0.21 g/L·h. Vacuum *in-situ* product recovery allowed the conduction of five consecutive batches lasting 209 hours total; glucose conversion, and IBE productivity increased to 66 %, 0.28 g/L·h, respectively. At the end of the fermentation, the condensate butanol concentration was 29 g/L de butanol, a much higher value compared to concentrations achieved inside the bioreactor.

Finally, sugarcane bagasse second-generation substrates were used to produce IBE. In fermentation of a cellulose hydrolysate containing 13 g/L lactic acid from microbial contamination supplemented with molasses, lactic acid was consumed by 92 % and butanol final titer achieved 7.9 g/L (0.28 g/g butanol yield). When this hydrolysate was mixed with overlimed hemicellulose hydrolysate and supplemented with molasses (35 g/L total sugar), *C. beijerinckii* exhaust glucose and utilized 38 % of sucrose, 31 % of xylose, and 70 % of lactic acid.

Suggestions for future works

1. The presence of fermentation inhibitors and different lignocellulose-derived sugars in the culture medium challenged IBE fermentation by *Clostridium beijerinckii* DSM 6423: sugar consumption was incomplete, and IBE productivity and final IBE titer were low. The addition of molasses as medium supplement improved fermentation performance. Therefore, the study of single-, repeated-, and fed-batch IBE fermentation using sugarcane bagasse as cell carrier for *Clostridium beijerinckii* DSM 6423 to produce IBE from sugarcane bagasse cellulose and hemicellulose hydrolysate medium supplemented with molasses, would be interesting. These experiments should be conducted inside the anerobic chamber, to provide the first knowledge on the behavior of immobilized cells in the presence of lignocellulose-derived fermentation medium.

2. Single- and repeated-batch IBE fermentation by *Clostridium beijerinckii* DSM 6423 using the fermentation system developed in this thesis with glucose P2 synthetic medium should be tested with varied vacuum parameters [time to begin vacuum sections, duration of vacuum sections, and frequency of vacuum sections (continuous or intermittent)], in order to find better process conditions to increase sugar consumption and IBE productivity.

3. Repeated-batch IBE fermentation by *Clostridium beijerinckii* DSM 6423 using

the fermentation system developed in this thesis should be tested with varied culture media to find better process conditions to avoid “acid crash”.

4. Varying the fermentation mode could avoid cell stress. In this work, we observed cell stress due to cell exposure to butanol, even when vacuum extraction was applied. Therefore, the conduction of IBE fed-batch and continuous fermentation by *Clostridium beijerinckii* DSM 6423 using the fermentation system proposed in this thesis could offer better fermentation results.

5. Previous experiments could serve as body of knowledge to perform IBE single- and repeated-batch fermentation by *Clostridium beijerinckii* DSM 6423 using the fermentation system developed in this thesis, with sugarcane bagasse cellulose and hemicellulose hydrolysate mixed with molasses as fermentation medium. These experiments would provide process conditions more similar to what we observe in the industries.

6. Finally, to avoid cell stress, IBE fed-batch and continuous fermentation by *Clostridium beijerinckii* DSM 6423 using the fermentation system developed in this thesis, with sugarcane bagasse cellulose and hemicellulose hydrolysate mixed with molasses as fermentation medium, should be tested.

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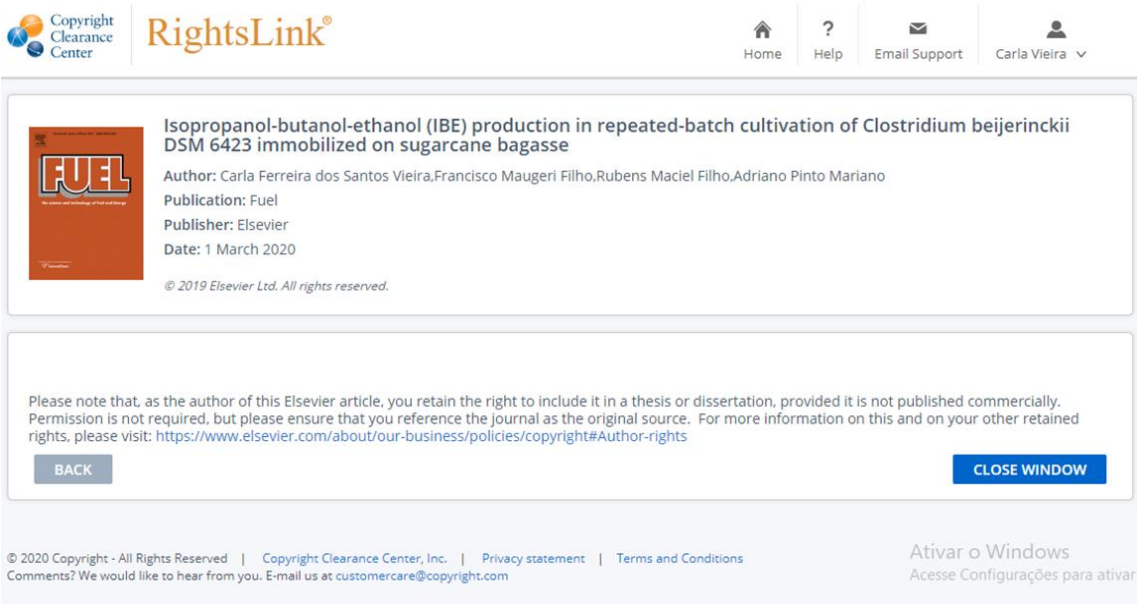

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APPENDIX B – Evaluation of the 3D printed cage-like structure geometry to trap the sugarcane bagasse as cell holding material for IBE fermentation.

INTRODUCTION

IBE fermentation by *Clostridium* bacteria is characterized by technical problems that reduce considerably the economic attractiveness of the process, i.e. high product inhibition, low yields and productivities, and elevated substrate costs. Attempting to increase cell concentration inside the bioreactor and consequently improve fermentation productivity, cell immobilization techniques using lignocellulose material as cell carrier have been successfully applied to IBE production (Vieira et al., 2019). However, as we discussed in Vieira et al., 2010 [section 2.1 (Figure 2.1.1 – p. 63)], lignocellulose materials such as sugarcane bagasse are less dense than water, and lose contact with the fermentation medium due to flotation, especially when fermentation gases are produced (bubbles carry the sugarcane bagasse to the surface). Therefore, the use of lignocellulose materials for immobilized fermentation requires a structure to trap the sugarcane bagasse inside the fermentation medium. This configuration creates a fermentation structure similar to fixed bed bioreactors.

While immobilized systems have been used to attack low productivity problems, *in-situ* product recovery is used to decrease product inhibition, allowing complete sugar conversion and consequent reduction in substrate costs. Several techniques that extract toxic products during the fermentation process were already applied in ABE (reviewed by Kolesinska et al., 2019) and IBE fermentation (reviewed by Vieira el al., 2019). Among them, butanol extraction by vacuum showed efficiency in butanol recovery during free cell ABE fermentation, increasing solvent productivity compared to control experiment (Mariano et al. 2011; Assumpção et al., 2018).

During the vacuum fermentation, vacuum directly applied in the bioreactor

decreases the pressure of the fermentation system, allowing volatile products to evaporate; then volatile vapors are condensed, and a more concentrated product stream is recovered. Products evaporation generates bubbles that circulate from the bioreactor downcomer to the riser, creating liquid streams (https://youtu.be/7pNo2Fcypr4) (Mariano et al., 2011). The hydrodynamic of this process is very similar to the process normally occurred in an air-lift bioreactor.

Kilonzo et al. (2010) published results concerning the hydrodynamic characteristics of an air-lift fibrous bed bioreactor where woven cotton was used as organic cell carrier. Researchers showed that spiral-wound fibrous bed can offer better homogenization and solid-to-liquid mass transfer performance in the combined system. Therefore, a spiral wound structure to trap the sugarcane bagasse as *Clostridium beijerinckii* DSM 6423 cell carrier for IBE production that offers a geometry which improve homogenization and solid-to-liquid mass transfer could be interesting, since fermentation products would be accumulated between the biofilms adhered to the sugarcane bagasse.

A fast and efficient way to produce different geometries to the spiral-wound structures to trap the sugarcane bagasse is the 3D printing. This technology produces objects in different shapes through deposition of materials (i.e. plastic, metal, ceramic, or even living cells) in layers, according the design defined in a computer-aided design (CAD). This process is also called additive manufacturing, rapid prototyping, or solid free-form technology. Among all 3D printing techniques, selective laser sintering (SLS), thermal inkjet (TIJ) printing, and fused deposition modeling (FDM) are the most used. The SLS technique is based on the utilization of powdered material for producing designed objects. A laser draws the shape of the object layers and fuses them until the 3D structure is formed. Normally, highly detailed structures are printed using this

technique since the degree of detail is limited only by the precision of the laser and the fineness of the powder (Hoy, 2013).

This work used 3D printing by selective laser sintering (SLS) to produce different geometries for the spiral-wound structure which was further used to trap the sugarcane bagasse as cell carrier inside the bioreactor for IBE production by *Clostridium beijerinckii* DSM 6423. The 3D printed structures were evaluated concerning mass transfer between the sugarcane bagasse and the liquid medium, attempting to improve solvents extraction, reducing butanol toxicity.

MATERIAL AND METHODS

The software SolidWorks was used to design all structures tested (Figure B.1). The structures were printed by rapid prototyping 3D printer SLS EOS FORMIGA P110, with nylon PA 2200 as material. Design and production of the 3D polymeric structure was conducted at the National Institute of Biofabrication - BIOFABRIS. All structures consisted of concentric cylinders of 12 mm thickness interspaced by 10 mm; its height was 80 mm and its diameter was 162.2 mm; the orifice diameter (which allowed the contact of the sugarcane bagasse with the medium) was 2 mm; three different geometries were tested (Figure B.1).

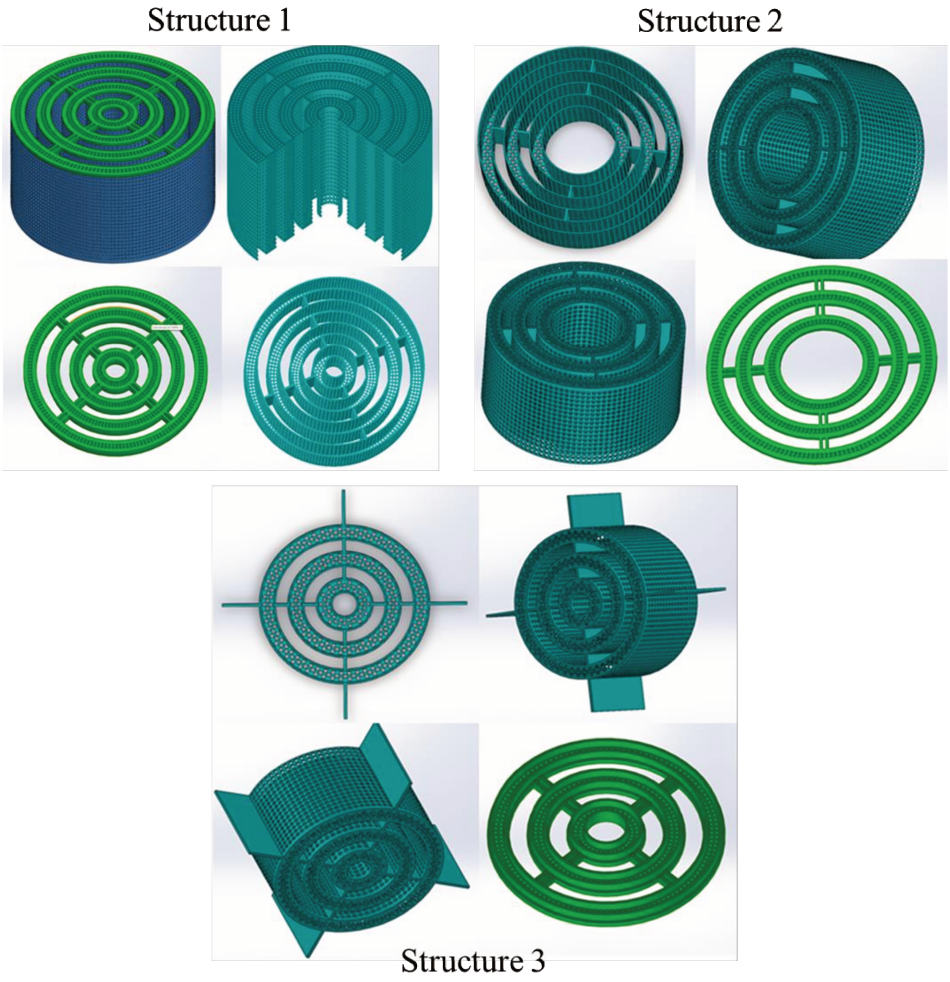

Figure B.1 – Structures designed on SolidWorks for 3D printing.

To evaluate the mass transfer offered by the 3D printed structure, we used violet crystal dye encapsulated in alginate. Sugarcane bagasse was placed inside the structure with small alginate spheres. This immobilization system was installed at the bioreactor and a 30 g/L ethanol solution at 35 °C (temperature of IBE fermentation) was used to mimic solvents produced during IBE fermentation. Once the vacuum system was working, ethanol evaporated generating bubbles and liquid streams. This movement inside the bioreactor stimulated the violet crystal dye decapsulation from the alginate spheres. Samples were taken every 30 min for 2 hours to measure the absorbance (580 nm) using a Laborglas LGI-VS-721N spectrophotometer. All tests were conducted in triplicates.

An aqueous solution containing 0.1 g/L violet crystal dye and 1.7 % (w/w) sodium alginate was dripped in a 10 % (w/w) calcium chloride solution under 100 rpm agitation to produce the small alginate spheres (5 mm diameter). The spheres were washed with distilled water to eliminate dye excess. The vacuum system and the bioreactor are described in sections 3.1.1.4 and 3.1.1.5. The control experiment consisted in placing the 3D structure inside the reactor with the 30 g/L ethanol solution without vacuum extraction and taking samples every 30 min for 2 hours.

RESULTS AND DISCUSSION

Figure B.2 shows all 3D printed structures used in the mass transfer experiments.

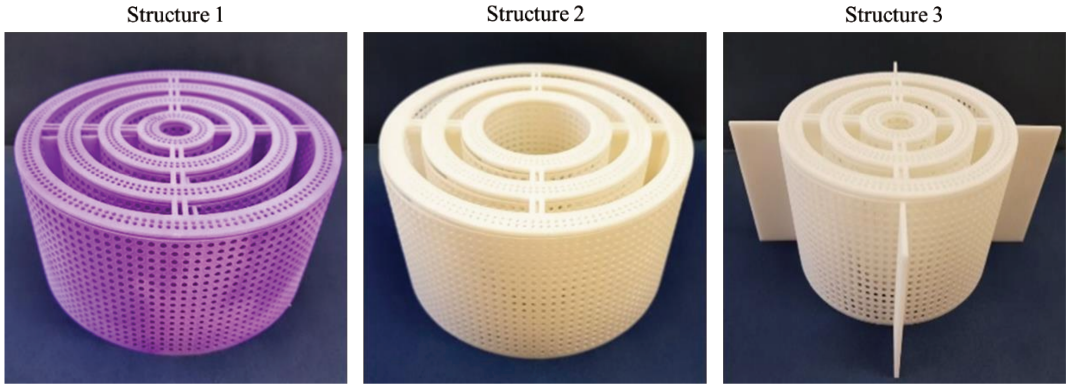

Figure B.2 – 3D printed structures used to trap the sugarcane bagasse and the alginate encapsulated crystal violet dye in the experiments of mass transfer.

Figure B.3a, b and c show the experiment results concerning mass transfer offered by each 3D structure geometry used to trap the sugarcane bagasse and the alginate encapsulated crystal violet dye spheres.

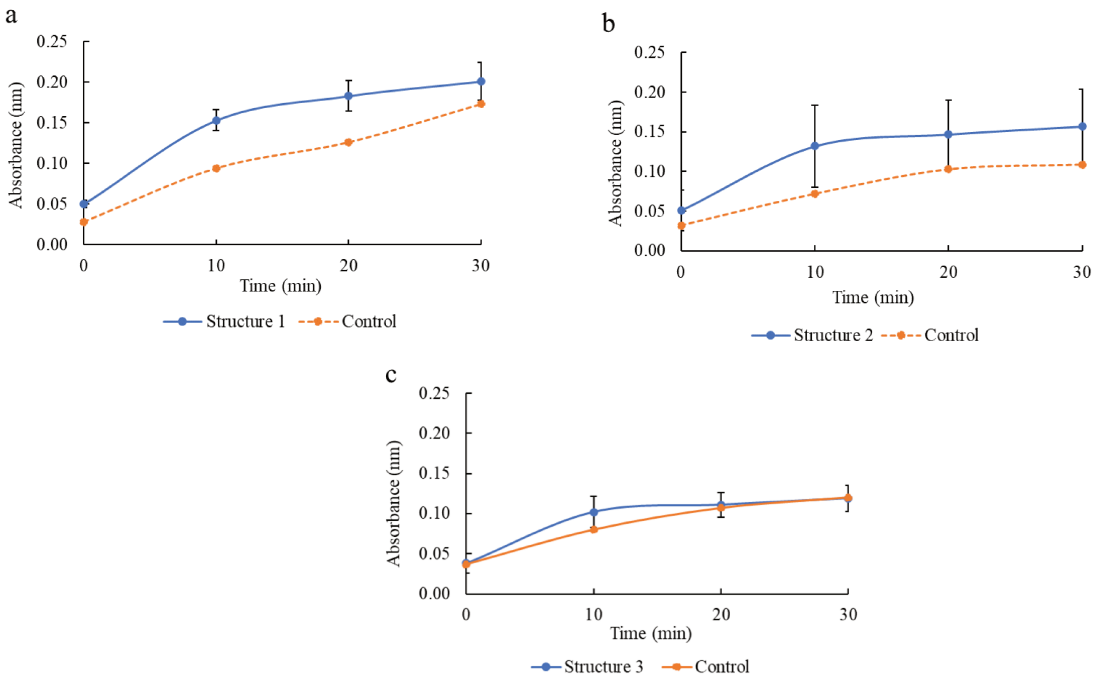

Figure B.3 – Absorbance results over time concerning mass transfer between alginate encapsulated crystal violet dye and the ethanol solution during vacuum extraction.

Structure 1 offered better mass transfer between the alginate encapsulated crystal violet dye spheres and the 30 g/L ethanol production since absorbance achieved during the experiment was higher compared to structures 2 and 3. Differently from the other two structures, structure 1 was composed by four concentric cylinders equally interspaced by 10 mm empty spaces; this geometry probably allowed higher contact between the spheres and the liquid media and a more turbulent circulation of the bubbles and the liquid, which could have improved homogenization inside the bioreactor.

CONCLUSIONS

The selective laser sintering 3D printing was an efficient strategy to produce the 3D structures to be used as trap for the sugarcane bagasse; the production was fast, precise and the material (nylon PA 2200) was resistant to autoclave and all mass

transfer experiments. Structure 1 was chosen to be used as trap for the sugarcane bagasse as cell carrier for *Clostridium beijerinckii* DSM 6423 to produce IBE. This structure offered better mass transfer results and the possibility of using higher amounts of sugarcane bagasse.

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