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Faculdade de Engenharia de Alimentos

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ADAPTIVE LABORATORY EVOLUTION OF C. SACCHAROPERBUTYLACETONICUM FOR ENHANCED TOLERANCE TOWARDS LIGNOCELLULOSIC INHIBITORS IN BUTANOL PRODUCTION

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Nothing in life is to be feared, it´s only to be understood (Marie Curie).

RESUMO

A produção de produtos de segunda geração (2G) inclui o butanol, que é uma molécula com um grande espectro de aplicações industriais, incluindo o uso como biocombustível. O butanol tem sido tradicionalmente produzido por rota química ou através de fermentação anaeróbica utilizando bactérias solventogênicas do gênero Clostridium spp. Estas bactérias são capazes de metabolizar grande variedade de fontes de carbono, incluindo a xilose o principal açúcar presente no hidrolisado hemicelulósico (HH). Entretanto a fermentação deste hidrolisado é severamente impactada pela presença de compostos inibitórios que afetam o crescimento dos microrganismos e a síntese de produtos de interesse. Assim, o objetivo deste trabalho foi gerar linhagens mutantes de Clostridium spp capazes de tolerar os principais inibidores presentes no hidrolisado hemicelulósico através de estratégias de evolução adaptativa (ALE). Os resultados obtidos mostraram que entre as linhagens estudadas inicialmente (*C. acetobutylicum*, *C. beijerinkii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*), o *C. saccharoperbutylacetonicum* apresentou o melhor desempenho fermentativo nas concentrações estudadas de açúcar inicial (10 g/L até 60 g/L), de modo que 50 g/L foi a condição que permitiu a máxima concentração de butanol (14.5 g/L) e rendimento de butanol (0.29 g/g). Além disso, a avaliação de quatorze inibidores no crescimento do *C. saccharoperbutylacetonicum* confirmou que o ácido acético (AA) e o 5-hydroxymethylfurfural (HMF) foram os principais inibidores que impactaram negativamente o crescimento, sendo o AA o principal inibidor a ser monitorado. A evolução adaptativa em laboratório (ALE) foi conduzida em concentrações crescentes de hidrolisado hemicelulósico e resultou em uma população evoluída (EP-40) após aproximadamente 130 gerações, na qual quatro linhagens (RAC-2, RAC-8, RAC-21 a RAC-25) foram isoladas. Estas linhagens evoluídas foram capazes de crescer na presença de ácido acético (5 g/L) e HMF (0.4 g/L), condição esta que inibiu completamente o crescimento da linhagem parental. Comparando as quatro linhagens evoluídas obtidas, a RAC-25 apresentou o melhor desempenho fermentativo, alcançando 16.7 g/L e 0.32 g/g de concentração de butanol e rendimento, respectivamente. A análise do genoma das linhagens evoluídas mostrou que somente a RAC-25 apresentou uma mutação deletéria no gene repressor transcricional da arabinose (*araR*), que pode estar diretamente relacionado a melhora na eficiência do consumo de açúcar (xilose) observado nos experimentos de crescimento em meio contendo ácido acético e HMF. Ademais, a mutação no anti-sigma fator I promoveu uma baixa expressão do *sigI* similar aos resultados obtidos com os outros mutantes. Os mutantes RAC-2, RAC-8 e RAC-21 não foram capazes de consumir eficientemente os

açúcares presente nos meios (com e sem inibidores) devido á mutação no sistema PTS como uma possível estratégia para melhorar o consumo de acetato. A análise da expressão gênica indicou a expressão de genes relacionados à bomba de prótons, biossíntese de prolina e chaperoninas, sugerindo um mecanismo integrado provavelmente coordenado pela repressão do *sigI* para tolerância aos inibidores. Em resumo, nós descobrimos um grupo de adaptações genéticas nos microrganismos para permitir o crescimento em meios contendo altas concentrações de AA e HMF. Estes resultados são importantes, pois trazem informações relevantes sobre genes relacionados a mecanismos de tolerância em bactérias do gênero Clostridium spp.

ABSTRACT

Production of second-generation (2G) products includes butanol, which is a molecule with a broad range of applications, including use as a biofuel. Butanol has traditionally been produced by chemical synthesis or anaerobic fermentation using solventogenic *Clostridium* spp. These solventogenic bacteria are able to metabolize a wide range of carbon sources, including xylose; the main sugar in the hemicellulosic hydrolysate (HH). However, the fermentation of HH is severely limited due to the presence of inhibitory compounds that affect microbial growth and product synthesis. Therefore, the aim of this work is to generate mutant strains of *Clostridium* spp., able to tolerate the main inhibitors present in hemicellulosic hydrolysate, via adaptive laboratory evolution (ALE). The results obtained indicated that among the strains studied (*C. acetobutylicum, C. beijerinkii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum*), *C. saccharoperbutylacetonicum* showed the best fermentative performance in a range of initial concentrations of 10 g/L to 60 g/L; with 50 g/L being the condition with the highest butanol titer (14.5 g/L) and yield (0.29 g/g). The adaptive laboratory evolution was performed in increasing concentrations of HH and resulted in an evolved population after around 130 generations; from which four strains (RAC-2, RAC-8, RAC-21 and RAC-25) were isolated. These evolved strains were able to grow in the presence of acetic acid (5 g/L) and HMF (0.04 g/L), a condition that completely abolished the growth of the wild type strain. In previous studies, we evaluated the effect of fourteen inhibitor compounds on *C. saccharoperbutylacetonicum* (parental strain) growth and confirmed that acetic acid (AA) and HMF negatively impacted growth; with AA being the most relevant inhibitor to be monitored. Comparing the four evolved strains, RAC-25 presented the best fermentative performance achieving 16.7 g/L and 0.32 g/g of butanol titer and butanol yield, respectively. Genome analysis of the evolved strains revealed that only the RAC-25 strain presented a deleterious mutation in the arabinose transcriptional repressor gene (*araR*); which could be related to the increased sugar consumption efficiency observed in the growth experiments. Moreover, the mutation in anti-sigma factor I promoted a down regulation of *sigI*, similar to other evolved mutants. The mutants RAC-2, RAC-8 and RAC-21 were not able to efficiently consume sugars present in the media (both with and without inhibitors) due to a mutation in the PTS system, as a possible strategy to improve acetate consumption. The gene expression analysis indicated high expression of genes related to H+ proton pumps (ATP synthases), proline biosynthesis (gamma phosphate reductase) and chaperonins (Grol); suggesting an integrated mechanism probably coordinated by the repression of *sigI* in order to tolerate the inhibitors. We have

discovered a set of genetic adaptations in bacteria to be able to grow on a culture medium containing a high concentration of AA and HMF. Our results are important in advancing information about possible genes related to tolerance mechanisms. We conclude that *sigI* and *araR* genes may be interesting targets to obtain robust strains with high tolerance to inhibitors presented in lignocellulosic hydrolysates.

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ABBREVIATIONS

μ_{max}: Maximum growth rate (h^{-1})

2G products: products produced from on lignocelullosic biomass

AA: Acetic acid

ABE: Acetone, butanol and ethanol

ALE: Adaptive laboratory evolution

araA*:* Gene responsible for production of arabinose metabolism transcriptional repressor AraR

ATP: Adenosine triphosphate

C5 sugar: Sugars with five carbons in composition

C6 sugar: Sugar with six carbons in composition

CA: *C. acetobutylicum* DSM 622

CAGR: Compound annual growth rate

CB: *C. beijerinckii* DSM 6422

cDNA: Complementary DNA

CL: *C. saccharoperbutylacetonicum* DSM 14923

CoA: Acetyl-coenzyme A

CO_2: Carbon dioxide

CCR: Carbon catabolic repression

CS: *C. saccharobutylicum* DSM 13864

DCW: Dry cell weight (g/L)

DT: Doubling time (h)

EP: Evolved Population

gDNA: Genomic DNA

GHG: Green house gases

grol: Gene responsible for production of 60kDa chaperonin

HH: Hemicellulosic hydrolysate

HMF: 5-hydroxymethylfurfural

HPLC: High-performance liquid chromatography

ICs: Inhibitory compounds

indel: Mutation of deletion type

MM: Mineral media

N_2: Nitrogen gas

P2: Semi-defined media used for cultivation of Clostridium spp

PPDP: Pilot Plant for Process Development

proA: Gene responsible to produce gamma-glutamyl phosphate reductase

ptsG: Gene responsible for production of PTS system glucose specific EIICB component

qs: Rate of substrate consumption (g sugar g $DCW^{-1}.h^{-1}$)

RCM: Reinforced Clostridium Media

RT-qPCR: Real-time PCR

RT: Relative tolerance

SEM: Scanning electron microscopy

***sigI*:** Gene responsible for the production of RNA polymerase sigma factor SigI

TRS: Total Reducing Sugars

WT: Wild type strain

$\mathbf{Y_{ABE/S}}$**:** Solvents yield (g solvents/ g sugars)

$\boldsymbol{Y_{but/S}}$**:** Butanol yield (g butanol/g sugars)

$\boldsymbol{Y_{X/S}}$**:** Biomass yield (g cell/ g sugars)

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1. INTRODUCTION

Brazil has been recognized as a major player in the bioethanol industry, predominantly being a part of production based on a biorefinery model, in which sugars present in sugarcane juice and molasses are converted to ethanol (first generation-1G) [1]. However, sugarcane processing results in a huge amount of by-product (bagasse), where the majority is burned by the ethanol industrial plants to generate steam and power. Alternatively, this biomass can be hydrolyzed and fermented to produce other products than ethanol, such as organic acids and other alcohols, a process known as second-generation (2G).

The lignocellulosic biomass is composed of two types of polysaccharides (cellulose and hemicellulose) interacting with lignin, a macromolecule responsible for the recalcitrance of biomass [2]. The main sugars released after biomass hydrolysis are hexoses (glucose, galactose and mannose) and pentoses (xylose and arabinose), that can be converted by the fermentation process into metabolites of industrial interest [3]. Recently, investments in research on second-generation bioethanol have encouraged the participation of some companies to produce ethanol from lignocellulosic material (bagasse and trash) [4]. However, the process faces relevant obstacles that need to be overcome in order for the industrial process to become economically feasible. The main challenges regarding 2G ethanol production are enzyme performance, reduction of enzymes costs, co-fermentation of C5 and C6 sugars, reduction of investment costs, reduction of process time and inhibitors produced during pretreatment [5]. The concept of biorefinery implies the conversion of all sugars present in biomass (sucrose, glucose, xylose, etc) into several products (biofuels, sugar, bio-oils, etc)[6], so that microorganisms that use pentose rich-fractions are important in a biorefinery context.

Clostridium spp. are solventogenic bacteria that can metabolize a broad range of sugars (starch, sucrose, glucose, xylose, cellobiose, arabinose) in order to produce solvents (acetone, butanol and ethanol), with butanol being the main product [7]. Butanol is an important molecule within many industrial applications (precursor for paints, polymers and plastics). Additionally, this compound is considered a “superior fuel” compared to ethanol due to a higher energy density and better performance [8].

The Global butanol market was evaluated at US$7.86 billion in 2014 with expectations to reach US$ 9.9 billion by 2020 (https://www.marketsandmarkets.com/PressReleases/n-butanol.asp). A study conducted by Mariano et al. (2013) evaluated the technical and economic aspects of integrating butanol production into a first-generation Brazilian sugarcane plant. The

researchers showed that higher prices of butanol in the market and the use of improved microorganisms are key elements to make investments in a biorefinery with butanol production more attractive [9]. This study indicated that the ABE process has the potential to be integrated into existing ethanol production plants; focusing on the use of the hemicellulose hydrolysate stream and using *Clostridium* spp. as the best naturally produced butanol platform.

Hemicellulosic hydrolysate is the pentose-rich stream usually obtained from the thermochemical pre-treatment step of the lignocellulosic biomass [10]. Usually, this liquid stream contains other compounds recognized as microbial inhibitors, generated during the pre-treatment. The presence of inhibitors and the high cost of the detoxification process to promote fermentability are challenges for the biotechnological routes to valorize the hemicellulosic hydrolysates. Several strategies comprising microbial adaptation, metabolic engineering and coupled with downstream processing steps have been extensively studied in the last 10 years, aiming at several chemical products including butanol.

2. REVIEW OF LITERATURE

BUTANOL

Currently, the world energy matrix is based on fossil fuel sources, 31.4% being from crude oil, 29.0% from mineral coal, and 21.3% from natural gas (International Energy Agency, 2014). Energy from fossil sources has constantly been rethought, mainly due to the fact that they are non-renewable sources, their high price fluctuations, and as well as their environmental impacts caused by greenhouse gas emissions [11]. As an alternative, biotechnology has been applied to develop greener fuels from renewable sources. Bioethanol is the main biofuel currently used around the world; in Brazil, bioethanol is produced on a large scale through fermentation of sugarcane juice and/or molasses feedstock using *Saccharomyces cerevisiae*, a process known as first-generation (1G) ethanol [1]. However, other types of liquid biofuels, such as isobutanol and butanol, produced by microbial fermentation are gaining attention in the industrial panorama [12].

Butanol has emerged as a "superior biofuel" compared to biodiesel and bioethanol. It presents a higher energy density and better performance when compared to bioethanol [13]. In addition, butanol can be directly used in existing combustible engines without prior modification. Moreover, butanol and its derivatives can be used in other important

applications, such as surface coatings, plasticizers and diluents; attesting to its versatility and increased market potential of the compound [14].

SOLVENTOGENIC CLOSTRIDIA METABOLISM

Microbial processes that utilize pentose-rich substrates are highly desired and must be considered in a biorefinery context. Microorganisms, such as *Clostridium* spp., display excellent potential for use in the 2G process, considering the broad substrate range available, in order to produce solvents (acetone, butanol and ethanol) through ABE fermentation. The development of the ABE fermentation platform began in the early 20th century; due to the high demand for acetone, used in the production of explosive cordite during the First World War (1914-1918) [15]. However, advances in the petrochemical industry in the middle of 20th century made the fermentation process less economically attractive [16]. Nevertheless, ABE fermentation has received renewed interest because of concerns about climate change, environmental pollution and exhaustion of fossil fuel sources [17].

In this regard, bacteria from the Clostridia genera, such as *C. acetobutylicum* and *C. beijerinckii*, are particularly interesting for biofuel production; as these are natural butanol producers with titers as high as 18 g/L [7]. Different species of butanol-producing Clostridia have been reported in literature: *C. beijerinchii* which produces butanol and isopropanol; *C. aurantibutyricum*, a producer of both acetone and isopropanol in addition to butanol [18]; and *C. tetanomorphum*, which produces butanol and ethanol (equimolar amounts) [19].

Clostridium spp. are normally spore forming, gram positive and anaerobic bacterium. This microorganism has the ability to consume a wide variety of substrates for growth, such as saccharides, oligosaccharides, polysaccharides, and various pentoses and hexoses, with the concomitant production of solvents [20,21]. They normally perform biphasic fermentation, the first phase known as acidogenesis, which is characterized by the degradation of sugars via glycolysis and associated with cell growth and acid production. The major end products are acetate and butyrate, along with the production of ethanol, hydrogen and CO_2. Due to acid production, the pH drops, which triggers the cells to switch the metabolism into a second phase; called the solventogenic phase, which is characterized by the re-consumption of the acids produced. These acids are converted into solvents, normally acetone, butanol and ethanol (ABE); with butanol being the main product [22,23]. For xylose metabolism, it has been supposed that the sugar is metabolized via the pentose-phosphate pathway, with xylose being converted to D-xylulose 5-P before entering into glycolysis [24] (Figure 1). It is reported

in literature that 0.411 g/g (butanol/glucose) is the theoretical maximum butanol yield achieved by *Clostridium* spp., based on stoichiometric reactions [25].

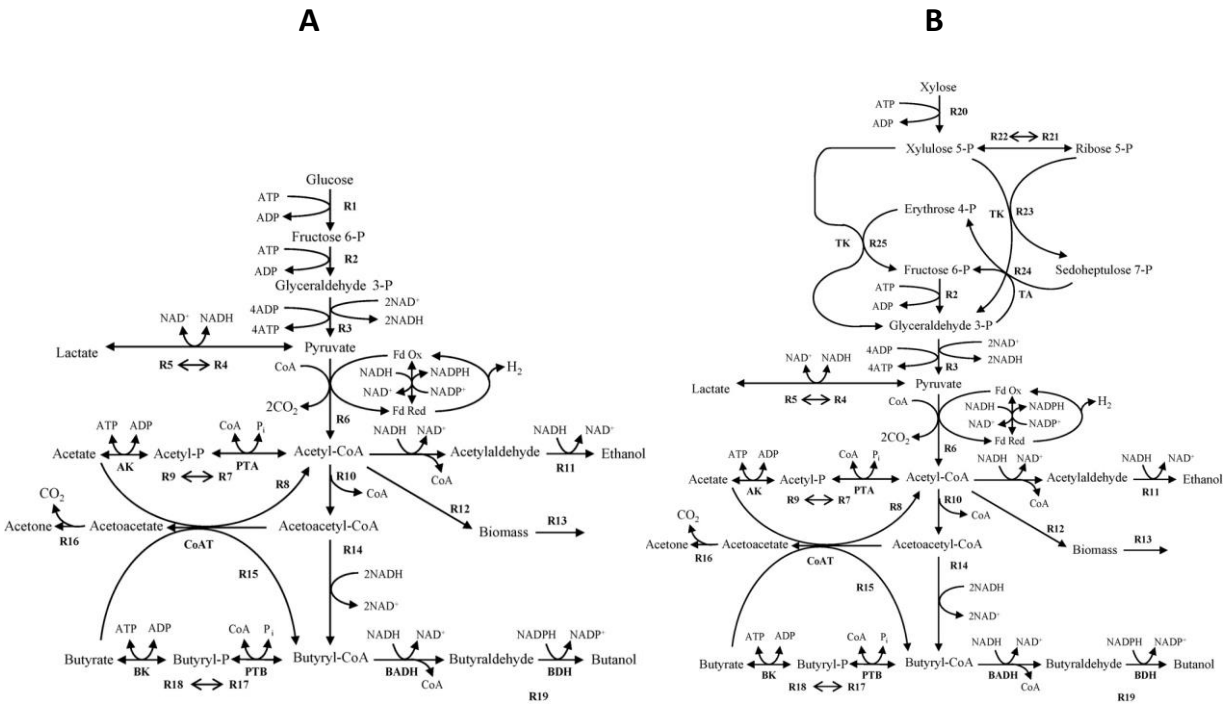

Figure 1. Central metabolism of *C. acetobutylicum* for acid and solvent production. In the acidogenic phase, acetate and butyrate are produced; while in the solventogenic phase acetone, butanol and ethanol are produced. A) glucose metabolism and B) xylose metabolism [26].

Recently, metabolic engineering technologies for butanol fermentation have been applied by researchers to develop and improve metabolic pathways in *Clostridium* species, as well as in other best-characterized hosts [27]. Yu et al. (2011) successfully engineered the *C. tyrobutyricum* ATCC 25755 strain (butyric acid production) to overexpress aldehyde/alcohol dehydrogenase (*adh*E2) from *C. acetobutylicum*, and achieved a butanol titer of 10 g/L [28]. Other studies have focused on *E. coli*, using metabolic engineering through the Keto-acid pathway, achieving a butanol concentration of 2 g/L [29]. Work on *Bacillus subtilis* and *S. cerevisiae* showed that these microorganisms could be interesting candidates for butanol production, because of their higher tolerance to solvents. However, reports have shown low butanol production (<1g/L) which is a drawback to the process [30,31].

CLOSTRIDIUM SACCHAROPERBUTYLACETONICUM

Substantial work has explored different *Clostridium* spp. for biobutanol production, with *C. acetobutylicum* and *C. beijerinkki* being the two main strains investigated [16,18,32]. However, other strains have recently gained attention in the biobutanol scenario, including *C. saccharobutylicum* and *C. saccharopebutylacetonicum*. In a work conducted by Magalhães et al. (2018), the researchers evaluated twelve *Clostridium* strains for biobutanol production using sugarcane straw hydrolysate as substrate. The results showed that *C. saccharobutylicum* and *C. saccharopebutylacetonicum* stood out amongst all strains. The first species exhibited a high capacity to metabolize sugars present in hydrolysate, consuming glucose and xylose simultaneously; and the second species presented a high biobutanol production (x g/L) [33]. Our research group has recently compared the fermentative performance of four *Clostridium* spp. (*C. acetobutylicum*, *C. beijerinkii*, *C. saccharobutylicum* and *C. sacchroperbutylacetonicum*) focusing on biobutanol production from lignocellulosic feedstock. The results indicated that between all evaluated strains, the *C. saccharoperbutylacetonicum* showed the best fermentative performance; achieving higher butanol yield ($Y_{but/s}$= 0.30 g/g on glucose and $Y_{but/s}$=0.25 g/g on xylose) during fermentation. Moreover, the highest butanol titer (14.5 g/L) was obtained when 50 g/L of sugars (93% xylose and 7% of glucose) was used to initiate the fermentation. Meanwhile, when fermenting non-detoxified sugarcane bagasse hemicellulosic hydrolysate, only 5.8 g/L of butanol was produced by *C. saccharoperbutylacetonicum*, indicating the difficulty in fermenting such complex media due to inhibitor content [34]. In another study conducted by Yao et al. (2016), the effects of representative sugars and lignocellulosic inhibitors on ABE fermentation with *C. saccharoperbutylacetonicum* was systematically evaluated. The results obtained showed that the strain was able to efficiently consume a wide range of sugars (glucose, cellobiose, xylose, arabinose and mannose), while degrading galactose slowly and incompletely. Regarding the effects of inhibitors on cells; ferulic acid, syringaldehyde and *p*-coumaric showed to be potent phenolic inhibitors, with *C. saccharoperbutylacetonicum* able to tolerate up to 0.8, 0.8 and 0.4 g/L of each previously described inhibitor, respectively. Furfural and HMF were not as toxic as phenolic compounds to the strain, with furfural being more rapidly converted into corresponding alcohol than HMF. The researchers concluded that the *C. saccharoperbutylacetonicum* can adapt to inhibitive conditions and produce more ABE than the control; demonstrating how robust the strain is for ABE production from lignocellulosic carbon sources [35]. Some work has also combined different substrates, such as molasses and hydrolysate, as a strategy to reduce the metabolic effects of inhibitors and increase nutrient supply for microbial cells [36–38]. Chacón et al. (2020) evaluated the use of molasses in

combination with hemicellulosic hydrolysate to produce biobutanol with *C. saccharoperbutylacetonicum*. For this, the crude hemicellulosic hydrolysate was fed after 24 h to a molasses fermentation containing 45 g/L of total reducing sugars. The results indicated that without the need of supplementing exogenous nutrients, the culture was able to efficiently ferment sugars present in the media (sucrose, glucose, fructose and xylose) in a formulation containing a diluted molasses to hydrolysate in volume ratio of 3:1. Under the condition studied, the strain could achieve high butanol yield (0.31 g/g) and titer (10 g/L) after 72 h of cultivation, suggesting that sugarcane molasses can be an efficient feedstock; enabling the production of biobutanol from sugarcane bagasse hemicellulosic hydrolysate [38]. Even with *C. saccharoperbutylacetonicum* being considered a robust strain for lignocellulosic inhibitor tolerance, some works have applied metabolic engineering to increase its capacity to withstand some inhibitory compounds and produce more solvents. In a work conducted by Jiménez-Bonilha et al. (2020), the researchers overexpressed efflux pump genes from *P. putida,* to enhance the tolerance of hyper-butanol producing *C. saccharoperbutylacetonicum* to fermentation inhibitors. The engineered strain, overexpressing the subunit *srpb,* showed an enhanced capacity to grow in media containing 17% more furfural or 50% more ferulic acid and produced around 14 g/L butanol, compared to control strains [39]. Other works have focused on engineering strains to improve sugar consumption to increase butanol titer and productivity in *C. saccharoperbutylacetonicum.* In this sense, Zhang et al. (2018) identified and studied the primary sucrose catabolic pathway in *C. saccharoperbutylacetonicum* through gene deletion using the CRISPR-Cas9 system. The mutant strains, with deletions of the transcriptional repressor gene, successfully alleviated CCR and enhanced ABE production by 24%. Additionally, overexpression of the endogenous sucrose pathway promoted better sucrose consumption and enhanced ABE production by 17.2%, 45.7%, and 22.5% compared to the wild type, with sucrose, mixed sugars or sugarcane juice as substrate, respectively [40]. The previously discussed works clearly suggest that *C. saccharoperbutylacetonicum* stands out as an interesting strain for industrial biobutanol production.

LIGNOCELLULOSIC BIOMASS AND INHIBITORS

The production of first-generation ethanol fuel generates a substantial amount of sugarcane bagasse, a by-product formed during the milling/extraction process. The Brazilian Sugarcane Industry Association (UNICA) reported that 653 million tons of sugarcane were processed at Brazilian distilleries and 176 million tons of bagasse was generated in 2017/2018 (Unica,

2017). Sugarcane bagasse consists of two types of polysaccharides: cellulose and hemicellulose. They are connected by a third component, lignin. Cellulose is a homopolymer of amorphous hexose, whereas hemicellulose is a heteropolymer of hexose, pentose and uronic acid; and lignin is a phenolic macromolecule [41]. Among them, cellulose and hemicellulose are the main biomass components that can be converted into metabolites of commercial interest through fermentation processes [42]. Sugarcane bagasse has been used mainly to generate steam and power in ethanol plants [43], or alternatively, it could be used for the production of ethanol or other biotechnological products with superior added-value.

Hemicellulose is a heteropolymer composed of linear chains containing branches formed by side hexoses (D-glucose, D-mannose and D-galactose), pentoses (D-xylose and L-arabinose); and can contain uronic acids such as D-glucuronic acid, D- galacturonic and methyl glucuronic [44]. The backbone of the hemicellulose fraction of sugarcane bagasse is composed of xylan (β- glycosidic linkages (1 → 4) and D-xylose residues, or L-arabinose) that connects with L-arabinose, glucuronic acid or methyl-glucuronic acid, forming the branches. Other substituents such as acetyl groups and hydroxycinnamic acids can be also found [45,46].

The hydrolysis of lignocellulosic biomass not only releases fermentable sugars but also several inhibitory compounds that can be classified into four groups: organic acids, aldehydes, ketones and phenols (Table 1). Acetic acid formed due to hydrolysis of present acetyl groups in hemicellulose, has been recognized as a potent inhibitor compound affecting microbial cells [47]. Furfural (2-furaldehyde) and HMF (5-hydroxymethyl-2-furaldehyde) are formed due to dehydration of pentose (hemicellulose) and hexose (glucose), respectively; have also been broadly studied regarding toxic effects to microbial cells [48,49]. Some of these compounds are reported to be toxic to several microorganisms during fermentation; and thus, may interfere in the biosynthesis of the desired product [50,51].

Table 1. Main inhibitory compounds produced during lignocellulosic biomass conversion.

Acids	Aldehydes	Ketones	Phenols
2-furoic acid	2-furfural	Acetoseryngone	Catechol
4-hydroxibenzoic acid	5-hydroximetilfurfural	Acetovanillone	Hidroxiquinona
Vanillic acid	4-hydroxibenzaldehyde		Eugenol
Syringic acid	Vanillin		Isoeugenol
4-hydroxicinamic acid	Seringaldehyde		
Ferulic acid			
Acetic acid			
Formic acid			

Hemicellulose hydrolysate (HH) fermentability is affected by the presence of inhibitor compounds (Table 1) and it is known that the hydrolysates' composition is strongly dependent on the biomass and on the pre-treatment steps performed [10]. The hemicellulose hydrolysis processes are usually performed under high temperatures and in some cases, acid addition as a catalyst resulting in several side reactions, such as sugar degrading reactions. Besides sugar, lignin is also affected by reaction conditions, generating free-phenolic compounds [52] (Figure 2).

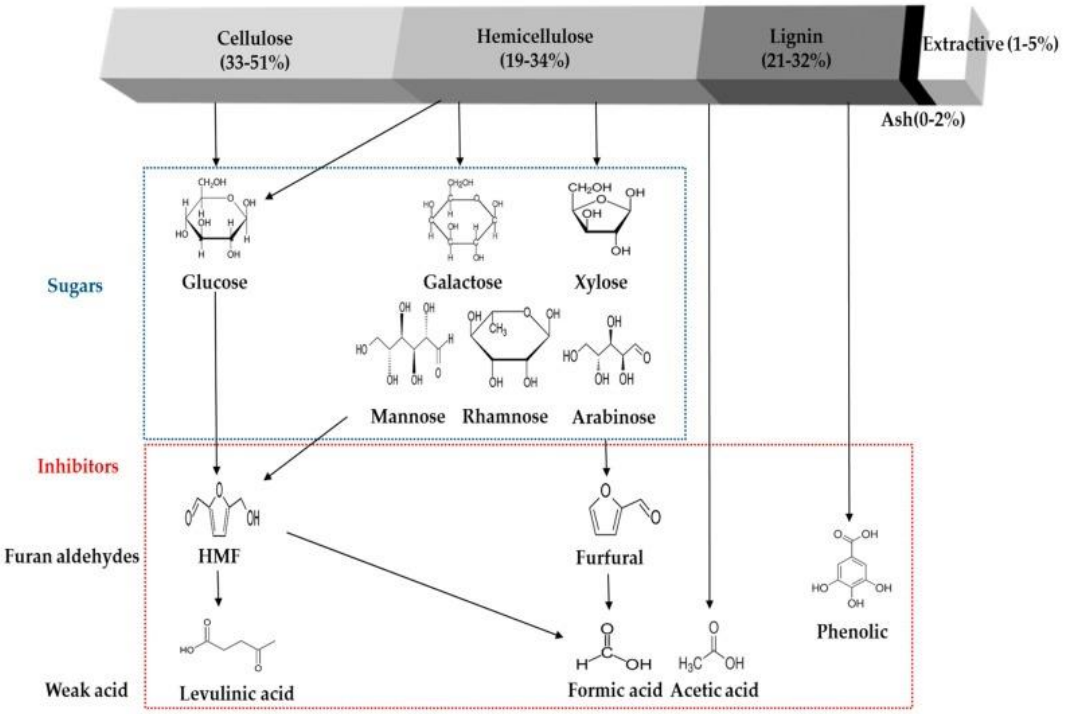

Figure 2. The chemical composition of lignocellulosic biomass and the main inhibitory compounds formed during the biomass processing [53].

In terms of the inhibitory action of these compounds on microbial cells, it has been reported that weak acids present in HH can inhibit fermentation through energy uncoupling or intracellular anionic accumulation [50]. Furfural and HMF on the other hand, when converted into their respective alcohols (that are less toxic to cells), scavenge reducing equivalents and as a consequence alter the redox equilibrium and the cofactor-dependent biosynthetic reactions are compromised [54]. Phenolic compounds have been described as powerful inhibitors to cells, altering the permeability and fluidity of biological membranes, and promoting cell membrane disruption [55].

In face of these observations, several studies on detoxification methods have been undertaken to reduce the toxic effects of inhibitory compounds derived from pre-treatment and neutralization processes; such as treatment with lime, peroxidases, and activated charcoal, as

well as adsorption techniques using ion-exchange resins [56,57]. More recently, other modern strategies have been applied to circumvent the problems promoted by inhibitors to a broad range of microorganisms. Liquid-liquid extraction is another promising alternative method applied for detoxification of inhibitory compounds, where the ionic liquids (extractant) are used for extraction [58,59] . Roque et al. (2019) evaluated two approaches for hemicellulosic hydrolysate detoxification: 1) liquid-liquid extraction (process 1) and 2) evaporation followed by liquid-liquid extraction, using 1- butanol, isobutyl acetate and MIBK as extractants. The results indicated that despite process 1 providing good extraction results; process 2 with MIBK showed to be a more promising detoxification process compared to process 1, with 85.4% total acetic acid extraction and 69% of total phenolics. The fermentability of hydrolysate using *S. stipitis* and *S. passalidarum* was also evaluated, and the results indicated that detoxified hydrolysate fermentation with *S. passalidarum* showed higher ethanol yield and productivity than *S. stipitis*[59]. Another method that has gained attention in the context of detoxification, is the use of different enzymes to reduce the toxic effects of inhibitors on microbial cells. Tramontina et al. (2019) developed a novel enzyme process with different redox activity enzymes to detoxify the hemicellulosic hydrolysate, and increase the fermentability using *C. saccharoperbutylacetonicum* and *S. stipitis.* The enzyme mixture composed of peroxidase (from Armocia rustica) together with superoxidase dismutase (from Coptotermes gestroi), were the most effective in detoxification of HH derived from sugarcane bagasse. Moreover the butanol production of the bacteria *C. saccharoperbutylacetonicum* and ethanol production by the yeast *S. stipitis* increased by 24x and 2.4x respectively, compared to untreated hemicellulosic hydrolysate [60]. It is important to highlight that the choice of detoxification method is directly influenced by the type of lignocellulosic biomass used, and the nature/concentration of inhibitors present in pretreated hydrolysate [61]. Detoxification processes involve a series of separation and purification steps that can strongly increase the overall costs of the process and limit the use of these sugars fractions. Although these unit operation steps are efficient for lignin derivative removal, they do present the inconvenience of their associated high costs.

ADAPTIVE LABORATORY EVOLUTION

In addition to detoxification techniques, other strategies based on adaptive laboratory evolution of microbial cells have been conducted to overcome the toxicity inhibitors. Adaptive laboratory evolution (ALE) or evolutionary engineering, is a strategy focusing on

microorganism improvement through natural selection [62]. ALE exploits the plasticity of microbial genomes by applying specific selective pressures in the laboratory environment that result in specific advantages (normally an industrially relevant trait) to the (eventually) evolved strain [63]. As a follow up, sequencing of the genomes of evolved strains and comparison with the parental strain allows for the identification of the important gene(s) responsible for the improved fitness. In practical terms, ALE involves basically two main systematic approaches: repeated batch cultivations or prolonged chemostat cultivations, both being performed in the presence of a pre-defined selective pressure [63].

Batch cultivations are normally performed in shake flasks or bioreactors, in which cells are cultivated in medium in the presence of a selective pressure, and an aliquot of culture is transferred into new flasks with incremental increases of the applied selective pressure in fresh medium, for a new round of growth [64]. The advantages of this type of methodology are the relatively easy setup and the low cost of equipment. However, the limited control of population density, growth rate, nutrient supply and environmental conditions (pH and dissolved oxygen) may lead to difficulties when using this methodology [63]. On the other hand, continuous cultivations (such as chemostats) allow for the control of doubling time (growth rate) and many environmental variables, and therefore a controlled system [65]. Controlling the growth rate is important to maintain the criteria throughout the evolution process, since this kind of experiment is naturally long-term and time-consuming [66]. In addition to regular ALE using specific selective pressures, such as inhibitors, some research has also applied different mutagenic compounds and radiation to boost the process to obtain evolved robust strains. Basically this strategy increases mutations rates, offering new options to increase mutation frequencies in evolving cultures [65]. Chemical mutagenesis induces reactions between chemicals and DNA; causing errors in base pairing, deamination of purines and transitions, transversions, and frameshift mutations [67]. ALE has normally been used to obtain mutants with relevant industrial traits when it is not simple to carry out genetic modifications on cells; due to the complexity of the process, including changes in metabolism and genome.

ALE has been applied to different microbial cells in order to obtain evolved strains tolerant to the main inhibitors present in hydrolysate-based media. In a work conducted by Koppram et al. (2012), long-term adaptation in repetitive batch cultures using a cocktail of 12 different inhibitors and long-term chemostat cultures using spruce hydrolysate was combined to improve inhibitor tolerance in a metabolic engineered xylose *S. cerevisiae* strain (TMB3400). The three evolved strains (RK60-5, RKU90-3 and KE1-17) displayed

significantly improved growth performance when compared to the parental strain, when cultivated in spruce hydrolysate. Beyond that, the evolved strains showed an increase in specific consumption rates of sugars and in specific ethanol productivity as compared to the parental strain [68]. In another work, Bonturi al. (2017) investigated the adaptation of the yeast *Rhodosporidium toluroides* to undetoxified sugarcane hemicellulose hydrolysate. The evolved strain had increased tolerance to inhibitors present in hydrolysate, and produced 41% more oil than the parental strain in xylose/glucose mixture [69]. Xia et al. (2018) used adaptive laboratory evolution to obtain an evolved strain of *Corynebacterium glutamicum* with a high capacity to tolerate the main inhibitors present in corn stover hydrolysate. In addition, the evolved strain showed an increased conversion rate of typical lignocellulosic inhibitory compounds (furfural, 4-hydroxybenzaldehyde and acetic acid), and an increased glutamic acid production compared to the parental strain [70]. Despite the works described above, we can note a scarcity of studies in the literature describing the application of ALE in Clostridia spp. towards increased inhibitors tolerance.

PROBLEM TO BE ADDRESSED

Despite the increasing interest for industrial butanol production from renewable feedstock, there are inherent process limitations, such as low butanol titer, solvent toxicity, fermentation stability and high operation costs that need to be overcome in order to make the process economically feasible [16]. Beyond the limitations mentioned above, the high costs associated with the feedstock (up to 66% of the total cost) has been singled out as an important drawback that significantly impact butanol production [71]. In this way, the use of low cost feedstock has been considered as an important alternative to reduce overall costs and improve sustainability of butanol production.

There is a considerable amount of five- carbon sugars (C5) from industrial by-products whose usage has not been properly addressed. Processes based on the biological conversion of these sugars into products of industrial interest have been sought as an alternative to the traditional chemical synthesis. By-product valorization is attractive due to the opportunity of low cost carbon and the sustainable aspect of environmentally-friendly processes. Furthermore, a significant number of chemical building blocks and intermediates may be obtained as metabolites of microorganisms from various biochemical pathways.

Ethanol is the main bio-product produced by sugarcane mills operating in Brazil. However, other compounds, such as butanol and organic acids, can be produced from the

fermentation of sugars (pentoses and hexoses); adding increased value to biorefineries and expanding product range. In fact, the concept of a sugarcane biorefinery implies the full use of sugarcane through co-generation of energy and fermentation of overall sugars; sucrose from the juice and glucose and xylose from bagasse [72]. However, the fermentability of hydrolysate is impaired by inhibitors that are generated during the pretreatment and hydrolysis processes, which may require diverse strategies (adaptive laboratory evolution, detoxification, metabolic engineering, etc.) to overcome this drawback.

Clostridium spp. are solventogenic bacteria that have the capability to metabolize several carbon sources for the production of ethanol, acetone and butanol. Starch, sucrose, glucose, fructose, galactose, cellobiose, xylose, arabinose and glycerol are some of the substrates utilized by this genus [73]. ABE processes for butanol production have largely been studied, especially those using *C. acetobutillycum* and *C. beijerinckii* strains; sequenced organisms with a properly annotated genome [74]. Fermentation performance levels of metabolically engineered *C. acetobutylicum* strains in glucose are around 10 to 17.8 g/L of butanol, compared with 5.5 to 11.7 g/L of butanol obtained by the control experiment; as reviewed by Lee et al (2008) [14].

In relation to lignocellulosic hydrolysates, some studies have exploited the use of these cheap low-cost substrates to produce biobutanol. In a recent work, cassava bagasse was efficiently fermented by *C. tyrobutyricum* overexpressing *adhE2*, producing a high butanol titer (> 15 g/L), yield (> 0.30 g/L) and productivity (~0.3 g/L.h)[75]. In a work conducted by Grassi et al. (2018), the authors evaluated the fermentative performance (butanol production, yield and productivity) of twelve *Clostridium* strains in sugarcane straw hydrolysate. The results revealed that among all the strains tested, *C. saccharobutylicum* DSM13864 showed the best performance; producing 10.3 g/L of ABE from a culture media composed of 79% pure hydrolysate and consuming 95% of available sugar [33].

Despite these studies previously discussed, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* are *Clostridium* species that are less investigated, evidenced by the low number of published works in the literature. In this context, the present work intends to further evaluate the fermentative performance of the main Clostridia spp. (C. *acetobutylicum* and *C. beijerinckii*), including other less studied strains (*C. saccharobutylicum* and *C. saccharoperbutylacetonicum*).

3. GOALS

GENERAL GOALS

The general goal of this work is to improve the *Clostridium* strain tolerance to the main inhibitors present in the hemicellulosic hydrolysates; using the strategy of adaptive laboratory evolution for the development of a second-generation butanol process.

SPECIFIC GOALS

The specific goals of this work are:

- To screen Clostridia strains that are reportedly good producers of butanol;
- To adapt and evolve a previously selected best strain, using adaptive laboratory evolution (ALE); to improve the strains ability to tolerate the main inhibitors present in hemicellulosic hydrolysate (HH);
- To characterize the evolved strains obtained by ALE, regarding fermentative performance and genomic profile in comparison to the parental strain;

4. RESULTS

The results are described in next two sections (4.1 and 4.2) in paper format; the first being published in 2019 and the second published in 2020.

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4.1 TOWARDS ENHANCED N-BUTANOL PRODUCTION FROM SUGARCANE BAGASSE HEMICELLULOSIC HYDROLYSATE: STRAIN SCREENING, AND EFFECTS OF SUGAR CONCENTRATION AND BUTANOL TOLERANCE

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Abstract

Robust strains are essential towards success of *n*-butanol production from lignocellulosic feedstock. To find a suitable strain to convert a non-detoxified hemicellulosic hydrolysate of sugarcane bagasse, we first assessed the performance of four wild-type butanol-producing *Clostridium* strains (*C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864, and *C. saccharoperbutylacetonicum* DSM 14923) in batch fermentations containing either xylose or glucose at 30 g L^{-1} as sole carbon sources. *C. saccharoperbutylacetonicum* was selected after achieving butanol yields as high as 0.31 g g^{-1} on glucose and 0.25 g g^{-1} on xylose. In a 48-h fermentation containing a mixture of sugars (93% xylose and 7% glucose) that mimicked the hydrolysate, *C. saccharoperbutylacetonicum* delivered the highest butanol concentration (14.5 g L^{-1}) when the initial sugar concentration was 50 g L^{-1}. Moreover, the selected strain achieved the highest butanol yield (0.29 g g^{-1}) on xylose-rich media reported so far. Meanwhile, *C. saccharoperbutylacetonicum* produced 5.8 g butanol L^{-1} (0.22 g g^{-1} butanol yield) when fermenting a non-detoxified sugarcane bagasse hemicellulosic hydrolysate enriched with xylose (30 g total sugars L^{-1}). Although sugars were not exhausted (4.7 g residual sugars L^{-1}) even after 72 h because of the presence of lignocellulose-derived microbial inhibitors, these results show that *C. saccharoperbutylacetonicum* is a robust wild-type strain. This microorganism with high butanol tolerance and yield on xylose can, therefore, serve as the basis for the development of improved biocatalysts for production of butanol from non-detoxified sugarcane bagasse hemicellulosic hydrolysate.

Keywords: Hemicellulosic hydrolysate; Xylose; Wild-type strain; *Clostridium saccharoperbutylacetonicum*; Butanol tolerance.

Introduction

The increasing global interest in biofuels, especially in those with fuel properties similar to gasoline, has created a market pull for advanced biofuels such as *n*-butanol (hereafter referred to as butanol). It has several advantages in relation to ethanol, such as higher miscibility with gasoline, higher energy density, lower volatility, and better biodegradability. However, technical difficulties still limit its production in large scale. Conventionally, bio-

based butanol is produced by solventogenic *Clostridium* strains in a strictly anaerobic process known as ABE (acetone-butanol-ethanol) fermentation. The primary challenges of this process are the high feedstock cost (60 − 70% of the production cost), the low butanol yield (~0.2 g g^{-1}), and the low productivity (< 0.2 g butanol L^{-1} h^{-1}) and titer (10 − 12 g butanol L^{-1}) due to the toxicity of butanol [43,71,76]. To overcome such limitations, recent studies have focused on the optimization of the ABE fermentation process and strain development using several metabolic engineering strategies [16]. In addition, substantial progress has been made in the use of low-cost agricultural wastes as feedstock to improve sustainability and reduce costs of butanol production [77,78].

The economics of butanol production can certainly benefit from existing sugarcane ethanol mills in countries such as Brazil, Colombia, India, and China because these facilities produce large amounts of bagasse. This lignocellulosic material is currently mainly used for energy cogeneration, but it could also be used to produce chemicals and fuels. Butanol is an interesting option because butanol-producing *Clostridium* strains can convert sugars derived from hemicellulose (arabinose and xylose). These sugars, on the other hand, cannot be metabolized by industrial *Saccharomyces cerevisiae* strains, thereby hampering their use for ethanol production. Since xylose is the primary sugar available in the hemicellulosic portion of bagasse, butanol can thus be an interesting alternative to add value to sugarcane bagasse [4].

However, the processing of lignocellulosic biomass, such as sugarcane straw and sugarcane bagasse, generates by-products that are inhibitory to microorganisms. The inhibitory compounds are organic acids (acetic, levulinic, and formic acids), furan derivatives [5-hydroxymethylfurfural (HMF) and furfural], and phenolic compounds [35,79]. These compounds are mainly present in the hemicellulosic hydrolysate, and they impact negatively the ABE fermentation [6]. As a result, studies have been searching for wild-type strains more efficient to convert sugars derived from the lignocellulosic fractions (straw and bagasse) of sugarcane. For example, Magalhães et al. [80] assessed twelve *Clostridium* strains for their ability to produce butanol from sugarcane straw hydrolysate. They found that *C. saccharobutylicum* can consume all sugars available in that feedstock. They also highlighted the high butanol-to-acetone ratio delivered by *C. saccharoperbutylacetonicum*. More recently, Grassi et al. [8] found that butanol production from sugarcane straw hydrolysate by *C. saccharoperbutylacetonicum* can improve when xylooligosaccharides are added to the fermentation. Other studies assessed ABE production from the overall hydrolysate (cellulosic + hemicellulosic) obtained from pretreatment and enzymatic hydrolysis of sugarcane bagasse [9,10].

However, rather less attention has been paid to finding butanol-producing *Clostridium* strains able to use the hemicellulosic hydrolysate of sugarcane bagasse as the sole carbon source. To fill this gap, in the first step of this study we assessed the performance of four wild-type strains (*C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum)* in producing butanol from xylose or glucose media. The strain with the highest butanol yield (*C. saccharoperbutylacetonicum)* was then further investigated to find the more suitable initial sugar concentration and to determine the tolerance of the strain to butanol. In the last step, we assessed the ability of the selected strain to produce butanol from a non-detoxified sugarcane bagasse hemicellulosic hydrolysate.

Material and methods

Microorganisms, culture maintenance, and inoculum preparation

The microorganisms used in this study (*C. acetobutylicum* DSM 6228, *C. beijerinckii* DSM 6422, *C. saccharobutylicum* DSM 13864, and *C. saccharoperbutylacetonicum* DSM 14923) were obtained from the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ). The strains were activated and propagated following the supplier's recommendations. Stock cultures were routinely maintained in 2-mL aliquots of 20% glycerol aqueous solution at -80°C. Inoculum was prepared in anoxic pre-sterilized Reinforced Clostridial Medium (RCM, Fluka, Sigma-Aldrich, Spain). Cells were cultivated anaerobically until the exponential growth phase (optical density, OD, at 600 nm = 1.0 – 1.5) in anaerobic chamber (Whitley DG250 Workstation, Don Whitley Scientific Ltd., West Yorkshire, United Kingdom). Inoculum size was 20 vol% in all fermentations. Morphological changes of the microorganisms were analyzed using microscopic inspection throughout the fermentation studies to monitor possible contaminations.

Screening of the Clostridium strains

In the first step of this study, the *Clostridium* strains were screened based on their ability to convert xylose and glucose, and their product yields. Fermentations were conducted in 100-mL screw capped bottles (triplicate) incubated still under N_2-enriched conditions in the anaerobic chamber. Fermentation medium (50 mL) contained 30 g L^{-1} sugar (glucose or xylose) and was supplemented with modified P2 medium (g L^{-1}): yeast extract, 5.0; KH_2PO_4,

0.75; K_2HPO_4, 0.75; NaCl, 1; $MgSO_4.7H_2O$, 0.4; $MnSO_4.H_2O$, 0.4; $FeSO_4.7H_2O$, 0.01; CH_3COONH_4, 4.3, para-aminobenzoic acid, 0.1, and biotin, 0.001. The medium was previously sterilized in autoclave at 121 °C for 20 min, while stock solutions containing $FeSO_4.7H_2O$, CH_3COONH_4, para-aminobenzoic acid, and biotin were filter-sterilized through a 0.22-µm nitrocellulose filter and subsequently added to the medium under sterile conditions inside a laminar flow hood. The initial pH was 6.4 and the cells were cultivated for 48 h at 35 °C (*C. acetobutylicum*, *C. beijerinckii*, and *C. saccharobutylicum*), and 30 °C (*C. saccharoperbutylacetonicum*). The optimal temperatures were found in preliminary tests (data not shown) based on the cultivation temperature ranges recommended by the supplier. Culture samples (2 mL) were collected at intervals (0, 3, 6, 24 and 48 h) and analyzed for cell growth (OD_{600nm}), concentration of sugar (glucose or xylose) and fermentation products.

Effect of initial sugar concentration on the selected strain

To assess the effect of the initial sugar concentration on the performance of the selected strain (*C. saccharoperbutylacetonicum*), the fermentation medium used in the screening step (section 2.2) was modified to contain a mixture of sugars (93% xylose and 7% glucose) with different initial concentrations (30; 40; 50; and 60 g L^{-1}). The sugars ratio was defined based on the typical composition of hemicellulosic hydrolysates after post-hydrolysis in H_2SO_4 solution (0.4 wt%) [81]. Fermentations were conducted (triplicate) in 300-mL bioreactors (Dasgip Box, DASGIP, Germany) at 30 °C and 200 rpm for 48 h. Working volume was 100 mL. The initial pH was adjusted to 7.0 using sterile 2 M NaOH solution. Prior to inoculation, the bioreactors were flushed with N_2 (100 mL L^{-1}, i.e. 1 vvm) for 2 h to create anoxic conditions before the start of each fermentation. During gas flushing, agitation and temperature were kept at 200 rpm and 30 °C, respectively. Flushing was stopped upon inoculation, and the positive pressure created by fermentation gases (CO_2 + H_2) sufficed to keep the anaerobic condition (confirmed by on-line measurement of dissolved O_2 concentration). Culture samples (2 mL) were collected at intervals (0, 3, 6, 24, 28 and 48 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugar (glucose and xylose) and fermentation products.

Growth and production kinetics of the selected strain

Kinetic parameters [maximum specific growth rate (μ_{max}), cells yield ($Y_{x/s}$), butanol yield ($Y_{but/s}$), and maximum rate of substrate consumption (q_s)] of *C.*

saccharoperbutylacetonicum were calculated considering the more suitable initial sugar concentration determined in the previous section. Fermentation was conducted in a 7-L bioreactor (New Brunswick Scientific Bioflo®/Celligen® 115, New Jersey, USA) at 30 °C and 200 rpm. Initial pH of the modified P2 medium was adjusted to 7.0 (using sterile 2 M NaOH solution), and it contained 50 g L^{-1} sugars (93% xylose and 7% glucose). Anaerobic conditions were maintained according to the procedure described in section 2.3. Culture samples (2 mL) were collected at intervals (0, 2, 4, 6, 8, 10, 12, 24, and 48 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugar (glucose and xylose) and fermentation products.

Tolerance of the selected strain to butanol

Fermentations to assess the tolerance of *C. saccharoperbutylacetonicum* to butanol were conducted in 100-mL screw capped bottles (duplicate) incubated still under N_2-enriched conditions in the anaerobic chamber. Cells were cultivated at 30 °C in RCM medium (30 mL) containing different initial butanol concentrations (3, 6, 12, 17, and 23 g L^{-1}). Cell growth (OD_{600nm}) was analyzed at different intervals (12, 24, 36 and 48 h) and was used to calculate the percentage of relative tolerance (RT) to butanol [82]. RT in each sampling time (*t*) is given by Eq. 1, in which control refers to fermentation without butanol addition.

$$RT\ (\%) = 100 \times \left(OD_{600nm,t} - OD_{600nm,t=0}\right) \times \left(OD_{600nm,t}^{control} - OD_{600nm,t=0}^{control}\right)^{-1} \quad (1)$$

Fermentation of sugarcane bagasse hemicellulosic hydrolysate

In the last step of this study, we assessed the ability of *C. saccharoperbutylacetonicum* to ferment sugarcane bagasse hemicellulosic hydrolysate. The sugarcane bagasse (50 wt% moisture content) was kindly supplied by Usina da Pedra, a sugarcane mill located at Serrana, SP, Brazil. The bagasse was dried at room temperature and processed as received, i.e. the bagasse was not washed to remove ashes and residual sugars. The bagasse was hydrothermally pretreated in the Pilot Plant for Process Development (PPDP) at the National Laboratory of Bioethanol Science and Technology – CTBE (CNPEM, Campinas, Brazil). The pretreatment was conducted in a 350-L Hastelloy C-276 reactor (POPE Scientific Inc., Saukville, USA) under the following conditions: 160 °C, 60 min, and solid-to-liquid ratio of 1:10. Upon completion of the pretreatment time, the reactor was slowly depressurized and cooled. The

pretreated liquor was collected and filtered (Nutsche filter, POPE Scientific, USA) and subsequently transferred to the acid-post-hydrolysis step (Figure. 3).

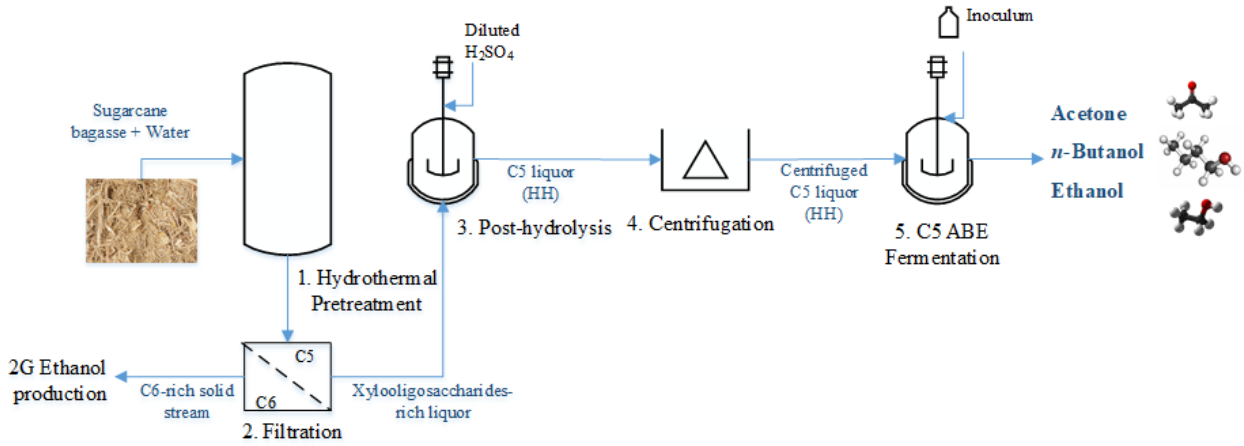

Figure 3. Schematic diagram of the production of sugarcane bagasse hemicellulosic hydrolysate (HH) and its use for ABE production.

The hydrolysis of remaining xylooligosaccharides was carried out in a 2-L stainless steel reactor (PARR Instrument Company, Moline, USA) using H_2SO_4 aqueous solution (0.4 wt%). This reactor was operated at 130 ºC and 200 rpm for 30 min. These conditions were previously determined [81] to complete the hydrolysis of the oligomers without increasing the amount of microbial inhibitory compounds. Subsequently, the suspension was centrifuged (9000 rpm) at 10 °C for 20 min. The resulting hemicellulosic hydrolysate containing approximately 17 g sugars L^{-1} was then filtered (0.22-µm polyethersulfone top filter; Nalgene, Rochester, NY, USA) for sterilization and removal of insoluble materials that would make it difficult to measure cell growth by absorbance. The filtered hydrolysate was stored in sterile glass bottles at -4 °C until use. The composition of the hemicellulosic hydrolysate is presented in Table 2.

Table 2. Composition of the sugarcane bagasse hemicellulosic hydrolysate obtained from the hydrothermal pretreatment, and its composition with xylose supplementation before inoculation.

Component	**Hemicellulosic hydrolysate** ($g\ L^{-1}$)	**Hemicellulosic hydrolysate + modified P2 medium + xylose (Fermentation medium)** ($g\ L^{-1}$)
Xylose	13.12	27.04
Arabinose	2.32	1.72
Cellobiose	0.63	0.47
Glucose	0.82	0.72

Total Reducing Sugars (TRS)	16.89	29.95
Acetic acid	4.17	3.36
Formic acid	0.18	0.11
HMF	0.12	0.10
Furfural	0.27	0.23
Syringaldehyde	0.07	0.06
p-Coumaric acid	0.21	0.18

Batch fermentation of the hemicellulosic hydrolysate was conducted in 300-mL bioreactors (Dasgip Box, DASGIP, Germany) (triplicate) at 30 °C and 200 rpm for 72 h. Anaerobic conditions were obtained according to the procedure described in section 2.3. The initial pH of the fermentation medium (240 mL) was adjusted to 7.0 using sterile 25% NH_4OH aqueous solution. Pre-sterilized hydrolysate was supplemented with modified P2 medium (described in section 2.2) and xylose to yield an initial xylose concentration of 30 g L^{-1}. Medium components were added to the hydrolysate under sterile conditions in a laminar flow hood. Composition of the resulting fermentation medium is presented in Table 2. Culture samples (2 mL) were collected at intervals (0, 3, 6, 20, 24, 30, 48, and 72 h) and analyzed for cell growth (OD_{600nm}) and concentration of sugars (glucose and xylose) and fermentation products.

Analytical procedures

Samples before chromatographic analysis were centrifuged (8000 rpm) at 4 °C for 10 min. The clean supernatant was transferred into 2-mL microtubes and stored at -10 °C until analysis. Before injection into the high-performance liquid chromatography (HPLC), samples were filtered using a 0.22-μm Millipore Millex-HV PVDF membrane filter. Solvents (acetone, butanol, and ethanol), sugars (glucose, xylose, and arabinose), and organic acids (acetic and butyric) were separated in a Bio-Rad Aminex® HPX-87H column (at 35 °C; 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}) and detected with refractive index detector (RID). Microbial inhibitory compounds (formic acid, HMF, furfural, syringaldehyde, and *p*-coumaric acid) were analyzed by reversed-phase HPLC, separated in a Thermo Scientific Acclaim® 120 C18 column (at 25 ^oC; 1:8 volume ratio of acetonitrile to water with 1 wt% acetic acid as the mobile phase at a flow rate of 0.8 mL min^{-1}), and detected with UV–Vis at 274 nm.

Culture growth was determined by measuring the optical density at 600 nm (OD_{600nm}) using a UV-Vis spectrophotometer (Thermo Scientific - Evolution 60S, Ann Arbor, Michigan, USA). In the kinetic studies (section 2.4), OD–dry cell weight relationships (Eq. 2 and 3) were

used to convert OD_{600nm} values to dry cell weight (DCW) per volume of culture medium (g L^{-1}) during growth and death phases.

$$DCW_{growth} = 0.4065 \times OD_{600nm} \quad (r^2 = 0.98) \tag{2}$$

$$DCW_{death} = 0.325 \times \left(OD_{600nm} + 3.20\right) \quad (r^2 = 0.99) \tag{3}$$

Results and discussion

Screening of the Clostridium strains

Among the four wild-type *Clostridium* strains assessed in this study, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* exhibited marked better performance. The former exhausted glucose in the glucose fermentation, achieving the highest ABE concentration [16.8 g ABE L^{-1} or 1.2 (A) + 10.9 (B) + 4.7 (E) g L^{-1}] (Figure 4). This strain also exhausted xylose in the xylose fermentation and produced 13.3 g ABE L^{-1} [0.5 (A) + 8.3 (B) + 4.5 (E) g L^{-1}]. Consequently, in both glucose and xylose fermentations, *C. saccharoperbutylacetonicum* achieved the highest ABE yield (0.42 g g^{-1} on glucose and 0.35 g g^{-1} on xylose; *Table 3*). The latter (*C. saccharobutylicum*) also produced ABE in relatively large concentrations: 15.2 g ABE L^{-1} (glucose fermentation) and 14.5 g L^{-1} (xylose fermentation). Notably, *C. saccharobutylicum* exhausted xylose in 24 h, while *C. saccharoperbutylacetonicum* required 48 h. However, *C. saccharobutylicum* was outperformed with respect to yields (0.29 g ABE g^{-1} on glucose and 0.28 g ABE g^{-1} on xylose). Interestingly, both strains delivered high ABE concentrations regardless of the carbon source (glucose or xylose). Moreover, the alcohols accounted for more than 90% of the total mass of solvents. Another advantage is that both strains presented relatively lower production and re-assimilation of acids, especially butyric acid. It suggests that butanol was synthesized through a different pathway in which the synthesis occurs via a direct route from acetyl-coenzyme A (CoA) and butyryl-CoA. This route was designated as the hot pathway by Jang et al. [83].

The other two strains (*C. acetobutylicum* and *C. beijerinckii*) were not able to exhaust either glucose or xylose. And they had poor solvents production (< 4 g ABE L^{-1}) (*Figure. 4*). As a result, yields were lower than 0.1 g ABE g^{-1} (Table 3).

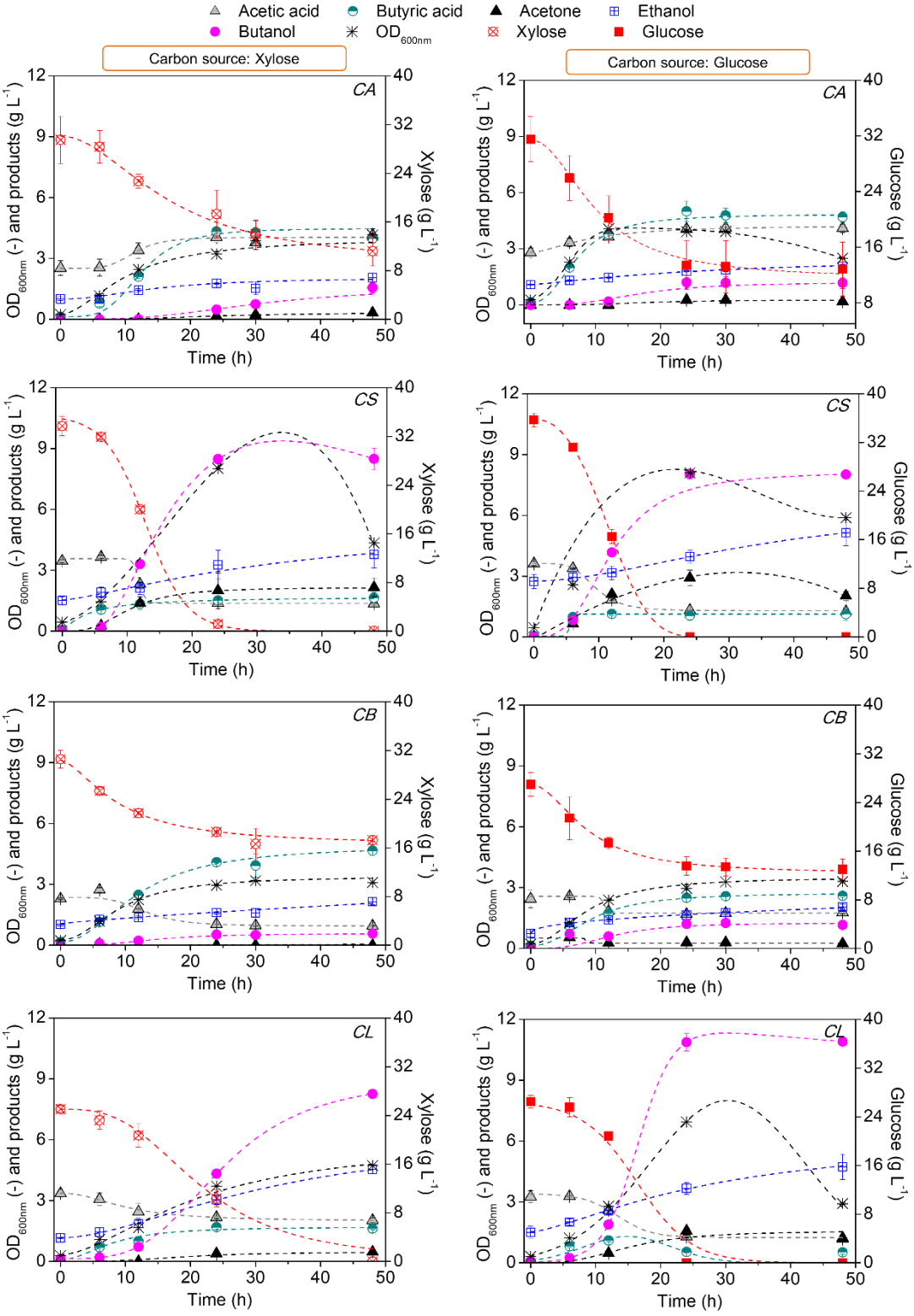

Figure 4. Production of ABE and acids, cell growth, and sugar consumption in ABE fermentations to screen the *Clostridium* strains. Xylose fermentation in the left column and glucose fermentation in the right column. CA: *C. acetobutylicum* DSM 622, CB: *C. beijerinckii* DSM 6422, CS: *C. saccharobutylicum* DSM 13864, and CL: *C. saccharoperbutylacetonicum* DSM 14923. Dashed lines represent a general tendency.

Table 3. Performance comparison of the Clostridium strains in glucose fermentation and xylose fermentation. Initial sugar concentration was approximately 30 g L−1 and fermentation time was 48 h.

Carbon source	**Strain**	**OD_{600nm}[(a)]**	**Yield[(b)] ($g\ g^{-1}$)**		**Productivity ($g\ L^{-1}\ h^{-1}$)**		**Residual sugar (%)**
			Butanol	*ABE*	*Butanol*	*ABE*	
Xylose	*C. acetobutylicum*	4.16±0.09	0.056±0.001	0.094±0.001	0.032±0.005	0.061±0.006	38.3±3.4
	C. saccharobutylicum	8.07±0.05	0.253±0.013	0.281±0.020	0.177±0.015	0.269±0.016	0.0±0.1
	C. beijerinckii	3.10±0.04	0.025±0.002	0.060±0.002	0.012±0.001	0.036±0.002	56.4±2.9
	C. saccharoperbutylacetonicum	4.73±0.01	0.247±0.017	0.351±0.012	0.169±0.003	0.250±0.003	0.0±0.0
Glucose	*C. acetobutylicum*	4.07±0.02	0.041±0.002	0.069±0.010	0.024±0.006	0.048±0.006	40.7±6.6
	C. saccharobutylicum	8.58±0.05	0.225±0.008	0.293±0.009	0.165±0.002	0.259±0.003	0.0±0.0
	C. beijerinckii	3.30±0.05	0.052±0.010	0.110±0.012	0.024±0.004	0.056±0.004	48.3±2.9
	C. saccharoperbutylacetonicum	6.95±0.02	0.310±0.012	0.422±0.012	0.225±0.003	0.317±0.003	0.0±0.0

(a) Maximum optical density in the fermentation. [(b)] Yield was calculated as grams of butanol produced per grams of sugar consumed.

Table 4. Effect of initial sugar concentration on the performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923. Fermentation time was 48 h.

Initial sugar ($g\ L^{-1}$) (93% Xyl + 7% Glic)	**OD_{600nm} (-)**	**Yield ($g\ g^{-1}$)**		**Productivity ($g\ L^{-1}\ h^{-1}$)**	
		Butanol	*ABE*	*Butanol*	*ABE*
30	7.23±0.27	0.22±0.03	0.28±0.04	0.15±0.03	0.18±0.02
40	7.67±0.03	0.26±0.08	0.32±0.06	0.24±0.04	0.29±0.04
50	10.80±1.53	0.29±0.07	0.35±0.06	0.30±0.06	0.36±0.08
60	9.14±0.28	0.27±0.04	0.35±0.03	0.30±0.06	0.39±0.08

One possible explanation for the poor performance is the fact that both strains produced relatively higher amounts of butyric acid during the growth phase up to 24 h. While this behavior is expected because acid production is coupled to the synthesis of one extra molecule of ATP to promote cell growth [26,84,85], the strains were not able to re-assimilate the acids to produce the solvents. As a result, acid accumulation may have inactivated microbial growth because of a sudden drop in the pH, a phenomenon known as "acid crash" [86,87]. This phenomenon was observed in other studies on ABE fermentation by *C. acetobutylicum* ATCC 824 [88] and *C. acetobutylicum* ATCC 39236 [17], for example. Nevertheless, further studies are needed to confirm our hypothesis and to elucidate the poor performance of *C. acetobutylicum* DSM 6228 and *C. beijerinckii* DSM 6422 observed in the present study.

For the next steps of this study, we selected *C. saccharoperbutylacetonicum* because this strain exhausted the sugars and presented the highest yields on both glucose and xylose fermentations. Yields are essential to the economics of commodity bioprocesses such as the ABE fermentation.

Effect of initial sugar concentration on *C. saccharoperbutylacetonicum*

The batch fermentations of *C. sacharoperbutylacetonicum* using a mixture of xylose (93%) and glucose (7%) at different initial sugar concentrations (30 to 60 g L^{-1}) demonstrated that the more adequate concentration is 50 g L^{-1}. While ABE concentration increased with sugar concentration, cell growth (maximum OD_{600nm} of 10.80) and ABE yield (0.35 g g^{-1}) were superior when the initial sugar concentration was 50 g L^{-1} (Table 4). Moreover, sugars were not exhausted when the concentration was higher than 50 g sugar L^{-1} (Figure 5). Other important advantages were improved solvents concentration (Figure 5) and butanol yield. Concentration of butanol (14.5 g L^{-1}) and ABE (18.0 g L^{-1}), and butanol yield (0.29 g g^{-1}) were higher than the values found in the xylose fermentation presented in the previous section. Remarkably, the

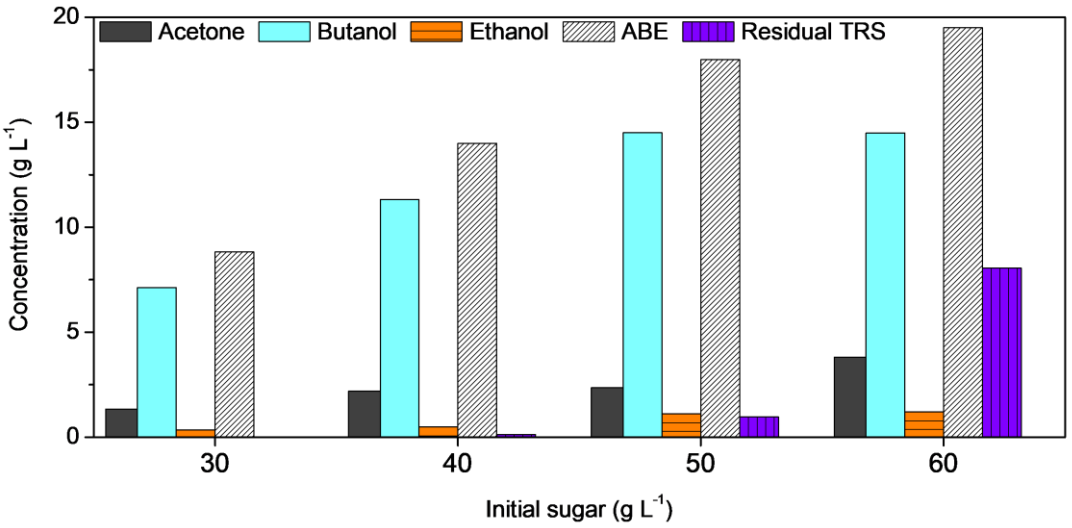

Figure. 5. Effect of initial sugar concentration on production of ABE and sugar consumption by *C. saccharoperbutylacetonicum* DSM 14923. Synthetic fermentation medium contained mixed sugars (97% xylose and 7% glucose). Fermentation time was 48 h.

butanol yield achieved by *C. sacharoperbutylacetonicum* when fermenting the sugar mixture at 50 g L^{-1} is, to the best of our knowledge, the highest value reported thus far for an ABE fermentation using xylose-rich media (Table 5). Consequently, the butanol-to-ABE ratio was as high as 0.80.

Table 5. Comparison of ABE production from xylose-rich media by *C. saccharoperbutylacetonicum* DSM 14923 with other wild-type Clostridium strains reported in various studies.

Strain	Carbon source	ABE ($g\ L^{-1}$)	ABE Yield ($g\ g^{-1}$)	Butanol ($g\ L^{-1}$)	Butanol yield ($g\ g^{-1}$)	Sugar consumption ($g\ L^{-1}$)	Butanol-to-ABE ratio	Ref.
C. sacharoperbutylacetonicum DSM 14923	93% Xylose + 7% Glucose	18.0	0.35	14.5	0.29	48 (from 50 g L^{-1})	0.80	This work
	Non-detoxified sugarcane bagasse hemicellulosic hydrolysate + xylose	7.11	0.26	5.85	0.22	30.0	0.82	
C. saccharoperbutylacetonicum DSM 14923	Detoxified rice bagasse hydrolysate	18.2	0.28	14.8	0.27	54.0	0.81	[18]
C. acetobutyllicum ATCC 824	Xylose	6.7	0.28	4.2	0.18	23.3 (from 60 g L^{-1})	0.62	[19]
C. beijeinckii NCIMB 8052	Xylose	7.9	0.24	6.8	0.22	32.7 (from 60 g L^{-1})	0.86	[20]
Clostridium sp. BOH3	Xylose	21.4	0.36	14.9	0.25	60.0	0.70	[21]
	Detoxified hemicellulosic hydrolysate	-	-	11.9	0.19	60.0	-	
C. acetobutylicum ATCC 824	Xylose	9.4	0.34	7.3	0.26	28.0	0.77	[93]
	Detoxified kraft black liquor	2.8	0.12	2.3	0.10	22.8	0.82	
C. saccharoperbutylacetonicum	Xylose	17.5	0.35	12.2	0.24	50.0	0.69	[35]
C. beijerinckii NCIMB8052	Xylose-rich medium	-	-	5.0	0.28	24.4	-	[94]
C. acetobutylicum DSM 1731	Xylose	5	0.25	3.6	0.18	20.1 (from 50 g L^{-1})	0.72	[95]
	Xylose-rich medium	9.5	0.27	6.3	0.18	35.1 (from 50 g L^{-1})	0.66	
	Mixture of barley straw hydrolysate and grain + xylose (89.9% xyl)	1.1	0.07	0.7	0.04	17.4 (from 49.8 g L^{-1})	0.64	
	Mixture of barley straw hydrolysate and grain + xylose (67.5% xyl)	6.9	0.35	4.2	0.21	19.8 (from 45.9 g L^{-1})	0.61	
C. acetobutylicum DSM 1731	Xylose (80% xyl) + starchy slurry	13.64	0.31	8.36	0.19	44.0 (from 50 g L^{-1})	0.61	[96]
	Xylose (80% xyl) + starchy slurry	4.84	0.22	3.08	0.14	22.0 (from 50 g L^{-1})	0.63	

(-) not reported

Growth and production kinetics of *C. saccharoperbutylacetonicum*

The 1-L fermentation to assess the kinetics of *C. saccharoperbutylacetonicum* confirmed the results obtained in the 100-mL fermentations (section 3.2), i.e. this strain can exhaust 50 g L^{-1} of a mixture of xylose and glucose in 48 h (Figure 6). The maximum rate of substrate consumption (q_s) and μ_{max} were 2.57±0.33 g sugar g $DCW^{-1}.h^{-1}$ and 0.37±0.01 h^{-1}, respectively (both parameters were calculated during the exponential growth phase). Interestingly, xylose and glucose were exhausted simultaneously. It was probably because glucose was in much lower concentration. When these sugars are in equivalent concentrations, previous studies found that *C. saccharoperbutylacetonicum* preferentially consumes glucose due to carbon catabolite repression [23, 27]. Upon consumption of both sugars in our kinetic experiment, butanol was the major product ($Y_{but/s}$ = 0.29 ± 0.04 g g^{-1}) and the cells yield ($Y_{x/s}$) was 0.14 ± 0.05 g g^{-1}.

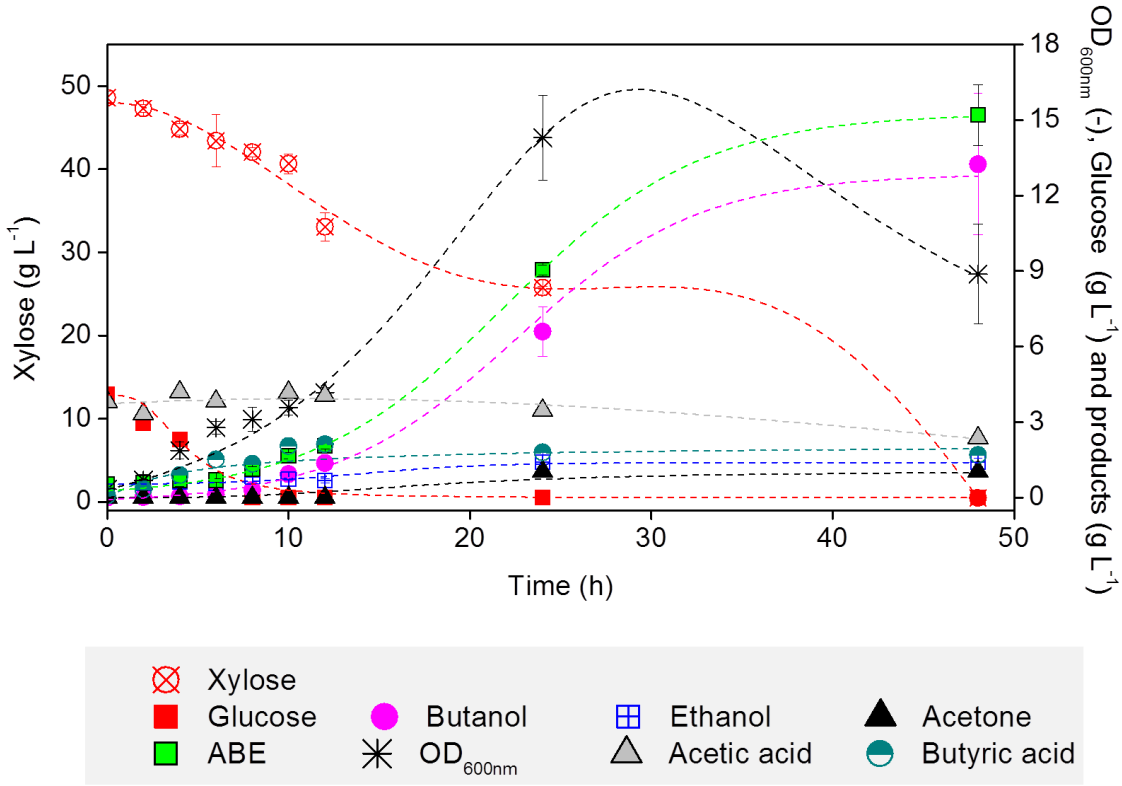

Figure. 6. Kinetics (production of ABE and acids, sugar consumption, cell growth) of *C. saccharoperbutylacetonicum* DSM 14923 cultivated in a synthetic fermentation medium containing mixed sugars (97% xylose and 7% glucose) at 50 g L^{-1}.

Tolerance of *C. saccharoperbutylacetonicum* to butanol

The inhibitory effect of butanol on growth of *C. saccharoperbutylacetonicum* was more pronounced when the culture was challenged by initial butanol concentrations equal to or higher than 12 g L^{-1}. When exposed to lower concentrations (3 and 6 g butanol L^{-1}) the cells needed 24 h to achieve a RT value of 100% (i.e. a cell growth equal to the control without butanol addition) (Figure. 7). In contrast, RT was 100% only after 48 h in the fermentation with 12 g L^{-1}. With respect to the concentrations of 17 and 23 g butanol L^{-1}, the cells were severely affected, and RT did not exceed 10%. This result agrees with the maximum butanol concentration (14.5 g L^{-1}) achieved in the experiments presented in section 3.2. Additionally, previous studies found that *C. saccharoperbutylacetonicum* can produce 16 g butanol L^{-1} from xylose (30 g L^{-1}) mixed with cellobiose (30 g L^{-1}) [27]. Thus, the maximum tolerance of *C. saccharoperbutylacetonicum* to butanol certainly lies in the range 15 to 17 g L^{-1}. These values are remarkably higher than the usual concentrations of 10 to 12 g L^{-1} obtained with wild-type strains [28], and this advantage can result in important gains in terms of energy consumption to distillate ABE [29].

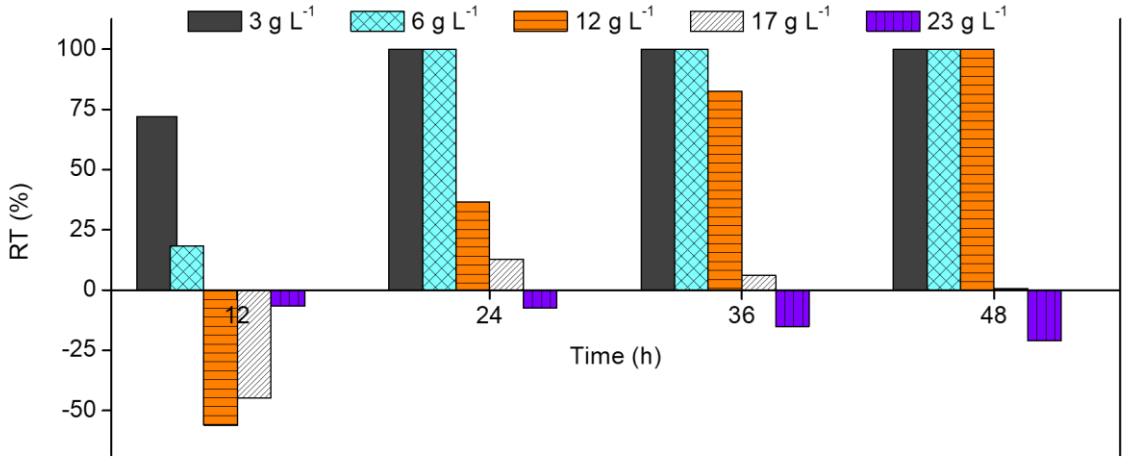

Figure 7. Inhibitory effect of different butanol concentrations on growth of C. saccharoperbutylacetonicum DSM 14923 during batch fermentation of 48 h. RT is the percentage of relative tolerance as defined in Eq. (1).

Fermentation of sugarcane bagasse hemicellulosic hydrolysate

Microbial inhibitory compounds found in the hydrolysate had detrimental effects on growth of *C. saccharoperbutylacetonicum* DSM 14923, sugar consumption, and solvents

production. If compared with the fermentation with the synthetic medium containing 30 g L^{-1} (section 3.2), the maximum absorbance (OD_{600nm}) decreased from 7.23 (synthetic medium) to 3.63 (hydrolysate medium). With respect to sugar consumption, *C. saccharoperbutylacetonicum* took 48 h to exhaust the sugars in the synthetic medium. In the fermentation of the hydrolysate, xylose was not completely consumed (4.7 g L^{-1} of residual sugars) even after 72 h (Table 6). Nonetheless, the low amounts of glucose (0.7 g L^{-1}) and arabinose (1.7 g L^{-1}) were exhausted in 3 and 20 h, respectively (Figure 8).

Table 6. Performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate supplemented with xylose.

Parameter	**Value**	
Fermentation time (h)	48	72
OD_{600nm} (-)	3.63±0.02	3.63±0.02
Butanol yield (g g^{-1})	0.21±0.02	0.22±0.02
ABE yield (g g^{-1})	0.24±0.04	0.26±0.04
Butanol productivity (g L^{-1} h^{-1})	0.07±0.02	0.08±0.03
ABE productivity (g L^{-1} h^{-1})	0.08±0.03	0.09±0.05
Residual sugars (%)	46.3±2.4	15.5±2.1

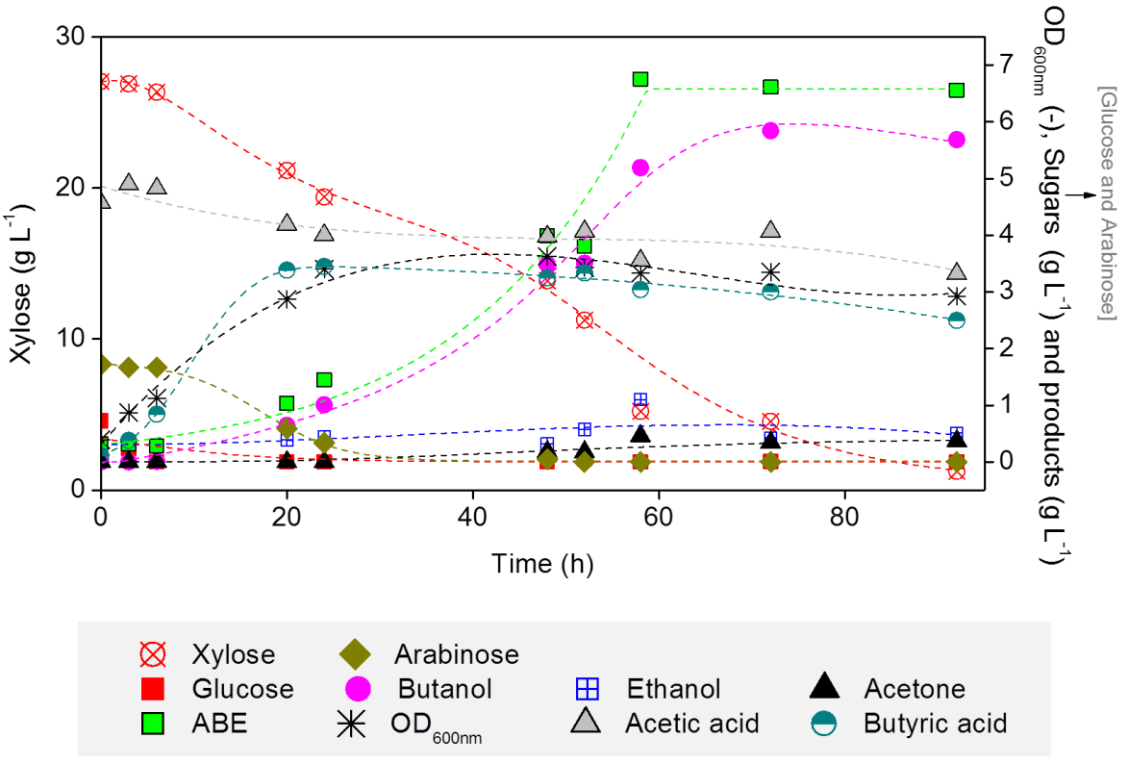

Figure 8. Production of ABE and acids, cell growth, and sugar consumption in the ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using sugarcane bagasse hemicellulosic hydrolysate supplemented with xylose**.**

The lower consumption of sugars impacted the solvents concentration. Butanol concentration was 5.8 g L^{-1} and lower than that obtained with the synthetic medium (7.1 g L^{-1}). Consequently, butanol productivity decreased from 0.15 (synthetic medium) to 0.08 (hydrolysate medium) g L^{-1} h^{-1}. Despite that, butanol yield was not affected (0.22 g g^{-1} in both synthetic and hydrolysate media) and the butanol-to-ABE ratio was also high (0.82).

The lower performance of ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate certainly resulted from synergistic effects of the inhibitory compounds. It means that their concentration (Table 2) would probably not be harmful if they were present individually. For example, acetic acid concentration in the hydrolysate medium (3.36 g L^{-1}) is similar to the initial concentration in the screening experiments presented in section 3.1 (Figure 4). Moreover, acetic acid concentration decreased throughout the fermentation with hydrolysate medium (Figure 8), indicating its consumption. In the case of *p*-coumaric acid and syringaldehyde, their concentration in the hydrolysate medium (0.18 and 0.06 g L^{-1}, respectively) are lower than the concentrations (0.4 g *p*-coumaric acid L^{-1} and 0.8 g syringaldehyde L^{-1}) that inhibited the growth of *C. saccharoperbutylacetonicum* in the studies conducted by Yao et al. [35]. They also report

ed that the strain tolerated concentrations of furfural and HMF of 2 g L^{-1} without having cell growth and ABE titer affected; moreover, the presence of HMF at concentrations between 1 and 3 g L^{-1} enhanced ABE titer. In the present study, furfural and HMF concentrations (0.23 and 0.10 g L^{-1}, respectively) were well below those thresholds.

However, if we had adjusted the xylose content in the hemicellulosic hydrolysate by evaporation (instead of adding synthetic xylose), this procedure would have increased the concentration of non-volatile inhibitors (mainly the phenolic compounds). This situation would certainly be even more aggravated if the hemicellulosic hydrolysate were concentrated by about three times to achieve the desired concentration of 50 g sugars L^{-1} determined by the fermentations with synthetic medium. On the one hand, the processing of a concentrated sugar stream would result in fewer fermentors and improved wastewater and energy footprints [29,30]. But on the other hand, these expected economic gains may not offset the costs related to evaporation and detoxification of the hemicellulosic hydrolysate. Thus, further techno-economic studies with focusing on this trade-off are needed.

Conclusions

The wild-type strains *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* presented a remarkable ability to ferment xylose-rich media. Notably, *C. sacharoperbutylacetonicum* attained the highest butanol yield (0.29 g g^{-1}) on xylose-rich media reported so far. This wild-type strain also presented high tolerance to butanol, achieving a maximum butanol concentration of 14.5 g L^{-1}. Our study also demonstrated that butanol production (5.8 g L^{-1}) by *C. saccharoperbutylacetonicum* using non-detoxified sugarcane hemicellulose hydrolysate is comparable to that (7.1 g L^{-1}) using synthetic medium and same sugar load (30 g L^{-1}). We conclude, therefore, that *C. saccharoperbutylacetonicum* can be used as the basis for the development of improved biocatalysts for production of butanol from sugarcane bagasse hemicellulosic hydrolysate.

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Conflicts of interest

None.

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4.2 ENHANCING ACETIC ACID AND 5-HYDROXYMETHYL FURFURAL TOLERANCE THROUGHT ADAPTIVE LABORATORY EVOLUTION

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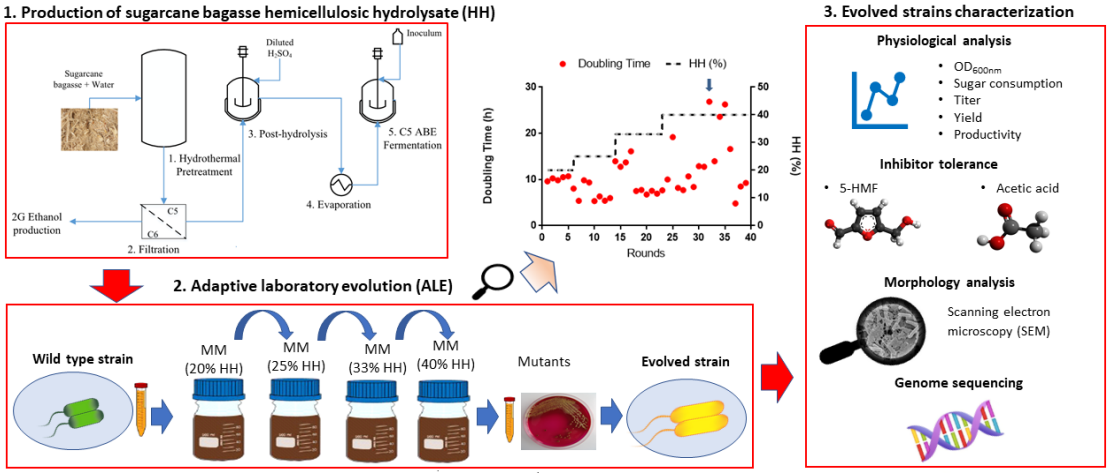
***corresponding author**

***Key-words*:** Adaptive laboratory evolution, Hemicellulosic hydrolysate, inhibitors tolerance, Clostridium spp, Butanol production, ABE process

Graphical Abstract



Abstract

In this study, adaptive laboratory evolution (ALE) was applied to isolate four strains of *Clostridium saccharoperbutylacetonicum* able to grow in the presence of hemicellulosic hydrolysate inhibitors unsupported by the parental strain. Among them, isolate RAC-25 presented the best fermentative performance, producing 22.1 g/L of ABE and 16.7 g/L of butanol. Genome sequencing revealed a deletion in the arabinose transcriptional repressor gene (*araR*) and a mutation in the anti-sigma factor I; that promoted a downregulation of *sigI*. Gene expression analysis indicated high expression of genes related to H^+-pumps (ATP synthases), proline biosynthesis (gamma phosphate reductase) and chaperonins (*Grol*), suggesting an integrated mechanism that is probably coordinated by the repression of *sigI*. Therefore, in addition to highlighting the power of ALE for selecting robust strains, our results suggest that *sigI* and *araR* may be interesting gene targets for increased tolerance toward inhibitor compounds relevant for lignocellulosic biofuels production.

Introduction

Butanol has emerged as a "superior biofuel" when compared to ethanol; offering advantages such as higher energy density, a less corrosive nature, higher octane number and higher hydrophobicity [85]. Additionally, butanol and its derivatives may be used in other important applications such as surface coating, plasticizing agent and as diluents; verifying the versatility and market interest of this compound [14]. Traditional butanol production has been based in the petrochemical industry; though recently, biobutanol production has received renewed interest due to its contribution to reducing the exhaustion of natural resources, environmental pollution and global warming [16]. However, biobutanol production is not economically competitive with the petrochemical-based butanol, due to the high cost of feedstock (usually molasses) and low butanol yield and productivity [16,71]. Thus, the opportunity for using low cost and abundant agro-industrial waste, which is mainly composed of lignocellulosic biomass as feedstock, opens a new chapter in the biobutanol development process [5,97–99].

Lignocellulosic biomass is the most abundant renewable carbon source on Earth, consisting of a composite material, primarily formed by two types of polysaccharides (cellulose and hemicellulose), and the complex aromatic compound lignin [99]. Given the recalcitrant nature of lignocellulosic biomasses, the access to whole sugars present in the plant cell walls can be carried out by two sequential hydrolysis steps; a thermochemical pretreatment to obtain the hemicellulosic hydrolysate, and an enzymatic hydrolysis of the cellulose fraction to generate the hexose-rich stream [10]. Hemicellulosic hydrolysate is a pentose rich liquor composed primarily of xylose [100] and also of inhibitory compounds formed during the pretreatment step, which can negatively affect the microbial cells and the fermentation process [79]. The presence of these toxic compounds affects cells in several ways: inhibition of cell growth by affecting glycolytic and fermentative enzymes, degradation of DNA, disruption of cell membrane and disturbance of ATP generation due to dissipation of the proton motive force [55,101]. In terms of inhibitory effects on living cells, it is well known that weak acids present in lignocellulosic-derived streams act by uncoupling energetic metabolism due to the effect of weak acids [79]. Phenolic compounds have been reported to be toxic even at low concentrations, reducing cell growth and ABE (acetone, butanol, and ethanol) production [35].

Detoxification methods such as the use of lime, peroxidases, activated charcoal, surfactant and ion-exchange resin adsorption [56,57,60,102] have been proposed to reduce the hydrolysates toxicity. Despite its effectiveness, the detoxification process involves a series of separation and purification steps that can sharply increase the overall cost of the process and limit its economic feasibility [47].

Strategies based on adaptive laboratory evolution (ALE) have been proposed as a valuable tool to enrich favorable genetic changes to obtain robust microbial cells that can withstand different inhibitor compounds. The concept of ALE or evolutionary engineering involves two approaches: repeated batch cultivation or prolonged chemostat with the presence of selective pressures to produce desired genetic variants [63]. Guo et al. (2013) obtained through continuous culture cultivation a high inhibitor tolerant mutant of C. *beijerinkii*; able to produce 12.9 g/L of ABE using non-detoxified hydrolysate from corn fiber [103]. Wang et al. (2017), applying a long term adaptive evolution strategy in non-detoxified corn stover hydrolysate, obtained a robust *Corynebacterium glutamicum* mutant with a high tolerance to various lignocellulose derived inhibitors [70]. The evolved strain increased the conversion rate of typical lignocellulose derived inhibitors (furfural, 5-hydroxymethylfurfural, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, and acetic acid) into less toxic compounds, better glucose consumption and an increase of 68.4% in glutamic acid production compared to the parental strain [70].

In this present work, we subjected *C. saccharoperbutylacetonicum* to adaptive laboratory evolution, to increase its tolerance to the main lignocellulosic derived inhibitors present in hemicellulosic hydrolysate. The evolved strains were characterized physiologically and morphologically. The genome was also sequenced to detect mutations that could bring valuable insights regarding the tolerance mechanism for further reverse metabolic engineering approaches.

Material and Methods

Strains and maintenance

The *C. saccharoperbutylacetonicum* (14923) isolate was acquired from the German Collection of Microorganisms and Cell Culture (DSMZ). The strain was activated and propagated following the supplier´s recommendations. Cultures of the strains were routinely maintained as a 2 mL suspension in glycerol (20% w/v) and stored at -80°C until experimentation.

Culture media preparation

Culture medium was prepared according to Zetty et al. (2019) [34]. Strain reactivation (pre-culture) was carried out in Reinforced Clostridia Medium (RCM, Fluka, Sigma-Aldrich, Spain), at 30ºC and inside an anaerobic chamber. For all fermentation experiments, strains were cultured in mineral medium (MM) that contained, in g/L: xylose, 55; yeast extract, 5; KH_2PO_4, 0.75; K_2HPO_4, 0.75; NaCl, 1; $MgSO_4.7H_2O$, 0.4; $MnSO_4.H_2O$, 0.4; $FeSO_4.7H_2O$, 0.01; ammonium acetate, 4.3 and supplemented with L-asparagine, 2; para-aminobenzoic acid, 0.1; and biotin, 0.001. For adaptive laboratory evolution (ALE) experiments, cultivation was performed in MM (as described above) containing around 55 g/L of xylose, supplemented with hemicellulosic hydrolysate (HH) at different percentages (v/v), according to each step of the evolution protocol.

Hemicellulosic hydrolysate production

Hemicellulosic hydrolysate production was obtained throughout the hydrothermal pretreatment, and carried out at the pilot plant facility of the Brazilian Biorenewables National Laboratory (LNBR/CNPEM, Campinas, Brazil) following the procedure described in detail from our previous work [81]. The liquor was concentrated 5-times in a pilot evaporator at the following operating conditions: pressure: 475 16 mbar; distillate: 80°C; temperature: 110°C-115°C. Thereafter, the concentrated liquor was centrifuged at 9000 rpm at 10ºC for 20 min. The pH was set to 6.5 using NH_4OH 25% (w/v). Finally, the HH was centrifuged at 8000 rpm for 30 min, filter-sterilized (0.22-μm polyethersulfone top filter; Nalgene, Rochester, NY, USA) for sterilization and removal of insoluble materials that would make it difficult to measure cell growth by absorbance. The filtered hydrolysate was stored in sterile glass bottles at −4 °C until use. Two batches of HH were produced and inhibitory compounds and sugars characterized and used in ALE experiments (Table 7). The same previously described protocol was used [81].

Table 7. Concentration of inhibitors and sugars present in two different batches of hemicellulosic hydrolysate used in ALE experiments.

Compounds	1st batch (g/L)	2nd batch (g/L)
HMF	0.06 ± 0.02	0.08 ± 0.01
Furfural	0.01 ± 0.01	0.02 ± 0.03
Acetic acid	3.38 ± 0.5	4.37* ± 0.8

Syringaldehyde	0.07 ± 0.02	0.08*± 0.02
Glucuronic acid	0.15 ± 0.1	0.90* ± 0.3
p-coumaric acid	0.08 ± 0.03	0.08 ± 0.01
4Hydroxybenzoic acid	0.01 ± 0.02	0.01 ± 0.03
Vanillic acid	0.009 ± 0.003	0.008 ± 0.002
Levulinic acid	0.18 ± 0.09	0.17 ± 0.1
Formic acid	0.35 ± 0.1	0.60* ± 0.2
Ferulic acid	0.16 ± 0.09	0.19* ± 0.08
Phenylacetic acid	0.21 ± 0.15	0.18 ± 0.07
Vanilin	0.08 ± 0.02	0.09 ± 0.01
Syringic acid	0.03 ± 0.01	0.04 ± 0.02
Glucose	1.51 ± 0.8	3.67 ± 0.5
Arabinose	12.9 ± 1.4	9.24 ± 0.8
Xylose	39.53 ± 1.9	47.85 ± 1.3
Total Sugars	53.96 ± 1.6	60.76 ± 1.8

*****Concentration higher in comparison to 1st batch.

Adaptive laboratory evolution (ALE)

An adaptive laboratory evolution (ALE) strategy was used to obtain robust *C. saccharoperbutylacetonicum* cells able to grow in media containing inhibitors derived from HH. For this purpose, a wild-type strain of *C. saccharoperbutylacetonicum* was submitted to serial batch cultivation in MM, supplemented with increasing concentrations of HH (from 20 to 40%, in v/v). The initial concentration of 20% of HH (HH-20) was based on preliminary data of wild type strain growth on medium containing different concentrations of HH (20%, 50% and 100%, v/v) (*Figure 1S*, Supplementary Material). All the fermentations were carried out in anaerobic chambers at 30°C with an initial pH of 6.5. First, the cells were cultivated in 20 mL of RCM the exponential phase was achieved (~1.5 OD_{600} nm), and then 2 mL was transferred into 18 mL of MM supplemented with xylose containing an initial concentration of 20% HH (v/v). Cells were cultivated in repetitive batch mode in this media until we observed a decrease in the doubling-time (DT). After no further decrease in this parameter, cells were transferred to another media containing a higher HH concentration than the previous media. For that, cells were harvested by centrifugation and resuspended in 2 mL of mineral media before being transferred to the new media with a higher HH concentration. Cells were cultivated in 25, 33 and 40% HH (v/v) along the ALE experiment. At 40% HH, a cultivation step without the selective pressure (no HH) was performed in between cultivations containing HH; the "on-off" strategy was proposed in a previous work [104]. The doubling time (DT) was used as the main parameter to evaluate the fermentative performance of the evolved population (EP) throughout

the cultivations rounds. Although the fermentative performance should be judged as the capacity to accumulate the desired product, we are assuming a strong relationship between growth and production; while production profile will be only evaluated for the selected clones. The DT was calculated according to the equation:

$DT = DurationCulture * \log(2)/\log(FinalConcentration) - \log(Initial\ concetration)$.

To isolate individual colonies from the EP-40 (40% HH), a 2 mL aliquot was cultivated in 15 mL of RCM until the exponential phase, and subsequently plated onto solid media (RCM). The largest colonies were selected, cultivated in RCM, and stored in 20% glycerol at -80°C.

Evaluation of mutants tolerance to acetic acid and HMF

The nine largest EP-40 colonies isolated from a solid plate (RCM) were evaluated for tolerance to acetic acid and 5-hydroxymethylfurfural (HMF). For this we carried out batch fermentations in 50 mL of MM containing xylose (60 g/L), acetic acid (5 g/L), and HMF (0.04 g/L) to compare the fermentative performance of the mutants and the wild type strain (WT). All fermentations were carried out in duplicate in an anaerobic chamber at 30°C. The initial pH was set to 4.95 and monitored off-line during fermentation using a pH meter (Metrohm). Cell growth was determined by measuring OD_{600}nm during cultivation. Samples were collected at 0, 24, 48, 72, 96, 120 and 144 h, and centrifuged at 8000 rpm for 10 minutes at 4°C. The clean supernatant was transferred into 2 mL microtubes and stored at -4 °C until further analysis. The concentrations of the solvents (acetone, n-butanol, and ethanol), sugars (glucose and xylose), and acids (acetic and butyric) were determined using high-performance liquid chromatography (HPLC) with a refraction index (RI) detector coupled to an Aminex HPX-87H column (BioRad). The mobile phase was 5 mM sulfuric acid with a flow rate of 0.6 mL/min at 35°C. In addition, the inhibitors furfural and 5-hydroxymethyl-furfural were analyzed using HLPC with a specific column UV detector (Acclaim 120 - C18 150 x 4.8 mm - Thermo). The column conditions were as follows: the mobile phase was acetonitrile in water (1:8) with 1% acetic acid and a flow rate of 0.8 mL/min. All samples were previously filtered using a 0.22 μm Millipore Millex-HV PVDF membrane filter. The culture growth was determined by measuring the optical density at 600 nm (OD_{600}nm) using a UV-Vis spectrophotometer (Thermo Scientific - Evolution 60S, Ann Arbor, Michigan, USA)[34].

Genomics

The total genomic DNA (gDNA) of four mutants selected from the 9 evaluated mutants were extracted using the Wizard Genomic DNA purification kit (Promega). The extracted gDNA was purified using PowerClean® DNA Clean-Up Kits (Mo Bio Laboratories) to ensure the sample quality. The DNA library was built by Nextera DNA sample preparation Kits (Illumina Inc., San Diego, CA, USA) and the fragmented sample was analyzed utilizing a Bioanalyzer (2100) with a 12000 DNA assay kit (Agilent). The libraries were pooled in equimolar ratios and subsequently submitted to paired-end sequencing on MiSeq instrument with one 150X150nt paired-end mode (Illumina platform); according to standard procedures of the Brazilian Biorenewables National Laboratory (LNBR/CNPEM, Campinas, Brazil), which resulted in about 300x average coverage of each sample.

NGS Data Analysis

The NGS pipeline consisted of the following steps: Fastq files → FastQC → Trimmomatic → BWA-MEM/Bowtie2 → Mpileup → Varscan → SnpEff [105–112]. For mutation analysis, the default setting in Bowtie2 was used for alignment and mapping [113]. The representative genome of *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) with taxonomy (ID) of 931276 (N1-4 (HMT) – ASM34088v1) was used as a reference genome for alignment. The results from the mapping were used to identify single nucleotide polymorphisms (SNPs), and insertions and deletions (indels) between the mutants and wild type. The results were further validated with the automatic Prokaryotic variant calling software Snippy. Genome annotation was done using Prokka and the aligned genomes and the SNP-indels were evaluated through viewing in IGV, Integrated Genome Browser [111]. Also, structural variants of the mutations were searched using the Delly software [112]. The mutations were also validated with different bioinformatics web platforms like Galaxy Melbourne and Patric. The types of mutations were classified using the SnpEff variant effect prediction software [109]. Further, the adverse of the mutations on protein sequences was predicted using Provean.

RNA Isolation

Cell cultivation was carried out in MM containing 55 g/L of xylose, 5 g/L of acetic acid and 0.04 g/L of HMF, with an initial pH of 6.5. The higher pH used in this experiment in comparison to challenge cultivation (pH of 4.9) was chosen to allow the wild type to grow

under acidic media conditions. For RNA isolation, 2 mL of culture was harvested and 4 mL of RNAprotect bacteria reagent (Qiagen, US) (1:2) was added immediately to stabilize and protect RNA from degradation. The material was mixed and incubated for 10 minutes at room temperature, centrifuged to obtain cell pellets, and stored at -80º C for the following steps. For cell wall lysis; 200 µL of TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 8) containing lysozyme (15 mg/mL) (ThermoFicher, USA) and 20 µL of proteinase K (20 mg/mL) were added, and cells carefully re-suspended. The material was then incubated at room temperature for 10 minutes. In continuation, we added 700 µL of RLT buffer (with beta-mercaptoethanol) and mixed vigorously, followed by the addition of 500 µL of ethanol. The RNA was purified using an RNAeasy mini kit (Qiagen Inc, CA) according to the manufacturer´s instructions. After the extraction, RNA was treated with Turbo DNAse free Kit (Invitrogen) following the manufacturer's protocol. RNA quality was analyzed using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US) and the concentration was determined using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA).

RT-qPCR for Quantify gene expression

Total RNA samples were used to synthesize the cDNA using the reagent Superscript II transcriptase reverse Kit (Invitrogen, USA) according to the manufacturer's protocol. The first round of end-point PCR was performed and the products were separated by agarose gel electrophoresis and purified with GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare, US). The amplicons were subjected to a 10-fold serial dilution (from 10^{-1} to 10^{-8}) and used to construct a standard curve. RT-qPCR reactions were performed according to Borin et al. (2017) along with the five best points of the standard curve and the cDNA samples from the experiments (see above), to keep the same conditions for standards and experimental samples (relative standard curve method) [114]. Primer sequences and genes analyzed are provided in Table 2. All RT-qPCR reactions were carried out in ViiA 7 Real-Time PCR system (Applied Biosystems, Life Technologies) using the following amplification conditions: activation for 10 minutes at 95°C followed by 40 cycles of denaturation (15 seconds at 95°C), annealing and extension (1 minute at 60°C). Data normalization was performed using quantification obtained from the housekeeping genes 1 and 2 (Table 8), and all reactions were conducted in triplicate. Statistical significance of the results was determined using analysis of variance ANOVA (Tukey´s test), with a significance level of 95% ($p< 0.05$). Analyses were performed using the GraphPrism 7.0 (GraphPad Software, San Diego, CA, USA).

Table 8. Primers used for RT-qPCR analyses of gene expression in the wild type and mutants (RAC-21 and RAC-25).

Gene name	Locus tag	Function	Primers 5´-3´(forward, reverse)
rpsL Housekeeping 1	CSPA_RS00900	Interacts with and stabilizes bases of the 16S rRNA that are involved in tRNA selection in the A site and with the mRNA backbone. 30S ribosomal protein S12; With S4 and S5 plays an important role in translational accuracy	GAGGTTGCTGAGAGGATTAATGC GATTCTACCTTTGGCCTTGGAA
psB Housekeeping 2	CSPA_RS06500	Ribosomal protein S2 belongs to the universal ribosomal protein uS2 family.	GAAGCAGGTGTACATTTCGGAC TACCTTCATCAGCTACTTGC
sigI	CSPA_RS16265	Sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released.	CTGAGATCGGTTCGTTCGGT GCTACTATTCTTGTAATAGGCAATC TC
proA	CSPA_RS00190	Catalyzes the NADPH-dependent reduction of L-glutamate 5-phosphate into L-glutamate 5-semialdehyde and phosphate.	AGTAAGCATGGGGCAAAATG ATTCGTGCAGCTTCTAGATC
atpD	CSPA_RS03060	Produces ATP from ADP in the presence of a proton gradient across the membrane.	TGAGGTTTCAGCGTTACTTGGA AGTCATCGGCAGGAACATATACTG
groL	CSPA_RS02180	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.	GGAAAAAGTAGGAAATGAAGGCG GCTTCTCCTTCAATGTCTTCAGC

Scanning electron microscopy

Morphology of bacterial cells (mutants and wild type) were analyzed using scanning electron microscopy (SEM). All strains were cultivated in individual batches on RCM in 15-mL shake flasks without agitation. All fermentations were carried out at 30°C inside an anaerobic chamber. Samples were taken at 24 h of cultivation and prepared according to the protocol established by Grassi et al. (2018) [115]. Samples were first filtered through 0.22 µm filters and then fixed for one hour (1 h) using 2.5% glutaraldehyde in 0.1% phosphate buffer (pH 7.4), followed by a washing step with 0.1M phosphate buffer (pH 7.4) for 15 minutes. Subsequently, the samples were dehydrated in a graded ethanol series (30% until 100%) and subjected to critical point drying followed by sputter-coating. Finally, samples were analyzed in a scanning electron microscope JSM 5800LV (Jeol).

Results and discussion

Adaptive Laboratory Evolution of *C. saccharoperbutylacetonicum* in the presence of hemicellulosic hydrolysate (HH)

ALE was carried out in batch cultivations with an initial concentration of 20% HH (HH-20) diluted in MM. This initial concentration was based on preliminary growth data of the wild type strain on medium containing different concentrations of HH (20%, 50% and 100%, v/v) (*Figure 1S*, Supplementary Material). The subsequent cultivations at increased concentrations of HH were applied when a reduction or a stabilization of the doubling time (DT) was observed along with the cultivation rounds. We then progressively increased the HH fraction in the MM. The progression of ALE for *C. saccharoperbutylacetonicum* under increasing concentrations of HH (25%, 33% and 40%, v/v) is depicted in Figure 9.

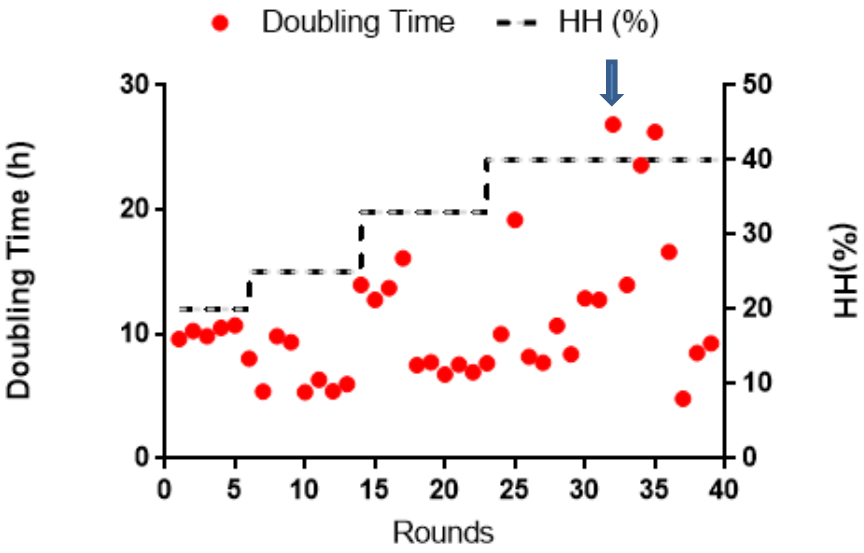


Figure 9. Progression of adaptive laboratory evolution (ALE) of C. *saccharoperbutylacetonicum* submitted to repetitive batch cultivations in high concentrations of hemicellulosic hydrolysate (HH). The arrow in the graph indicates the second batch of HH used in media composition for ALE**.**

At HH-20 cultivations, five repetitive batch cultivations (rounds) were performed, encompassing 13 generations. In this first step, cells were able to grow at a fairly constant DT over the five rounds, suggesting that at 20% concentration (HH-20) the inhibitor titers did not severely impact microbial cells. The evolution experiment was continued by changing to a medium containing 25% HH (HH-25), starting with the evolved population (EP-20). Under this condition, eight rounds were performed for a total of 27 generations. The DT progressively decreased during the cultivations, with a reduction of 30% of DT in the last four rounds, in comparison to the first four rounds. Subsequently, we continued the evolution protocol in the presence of HH-33, starting with EP previously obtained (EP-25). After nine rounds and 22 generations in this condition, we were able to obtain an evolved population (EP-33) with a reduction of 48% in DT in the last five rounds compared to the first four rounds. In the last step of ALE, we challenged the EP-33 with HH-40. Cells were submitted to 17 rounds of cultivation, comprising of 66 generations under this condition. The results indicate that until round nine, the DT was practically unchanged; however after round ten, this parameter increased until round 14. This fact can be explained by the use of another HH that was obtained using the same pre-treatment protocol described. However, this new batch contained a higher concentration of inhibitors compared to the first, and consequently appeared to be more toxic to the cells at the same concentration (HH-40) (Table 7). To facilitate data analyses, we can consider cultivations from round one to nine performed with the first batch of HH as separate;

and from round ten to seventeen as another that utilized the higher inhibitor HH batch. In this case, we can divide the ALE with HH-40 into two parts. In the first, cells were evolved over 24 generations and a significant improvement in DT or final OD was not observed. In the second, we observed an increase of DT in the initial rounds due to the higher inhibitor concentration of the new HH batch, followed by a substantial decrease in DT. After approximately 130 generations, the adopted ALE strategy resulted in an evolved population (EP-40) with an improved fitness in HH supplemented media; with a 26% reduction in DT, in comparison to the cultivations with HH-20 and HH-40 (last three rounds). Finally, in order to obtain isolates from this EP-40, cells were plated onto solid RCM medium, and large colonies were selected and stored (Figure 10).

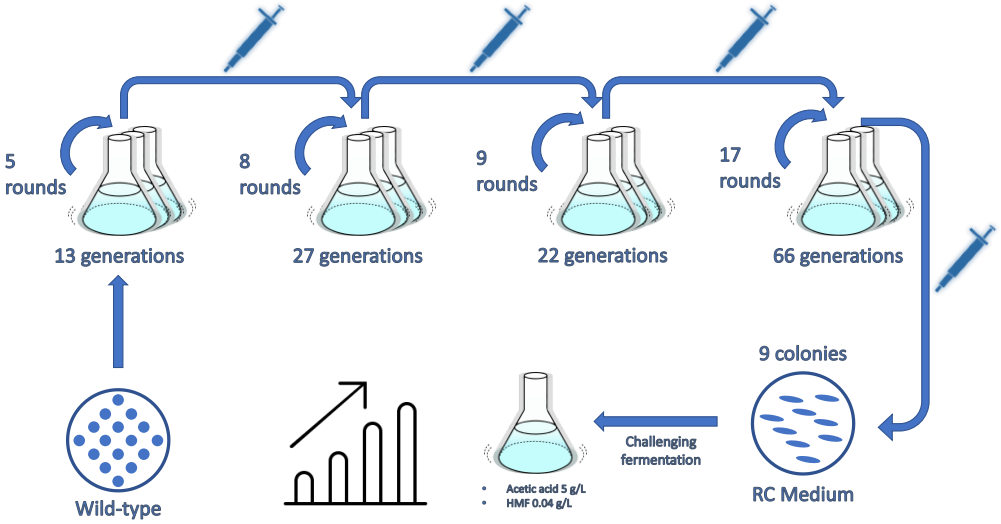


Figure 10. Workflow of the ALE strategy used to obtain robust strains of C. *saccharoperbutylacetonicum*, showing the number of rounds, generations and relevant colonies picked for further steps**.**

Evaluation of the evolved isolates toward acetic acid and HMF

The isolation of single EP-40 colonies from a solid plate (RCM) resulted in 9 colonies (mutants); to be evaluated for tolerance to acetic acid and HMF, previously identified (*Table 1S, Table 2S and Figure 2S*, Supplementary Material) as the inhibitors that most negatively impact *C. saccharoperbutylacetonicum* growth. The isolates from the ALE experiment (EP-40 isolates) were evaluated in MM containing acetic acid and HMF, and by comparing their fermentative performance with the performance of the parental wild type strain. The concentration of inhibitors (5 g/L of acetic acid and 0.04 g/L of HMF) used in this work were

higher than those present in the medium with HH-40. Cultivation under the presence of acetic acid and HMF showed that not all isolated mutants were able to grow under such conditions (Figure 11).

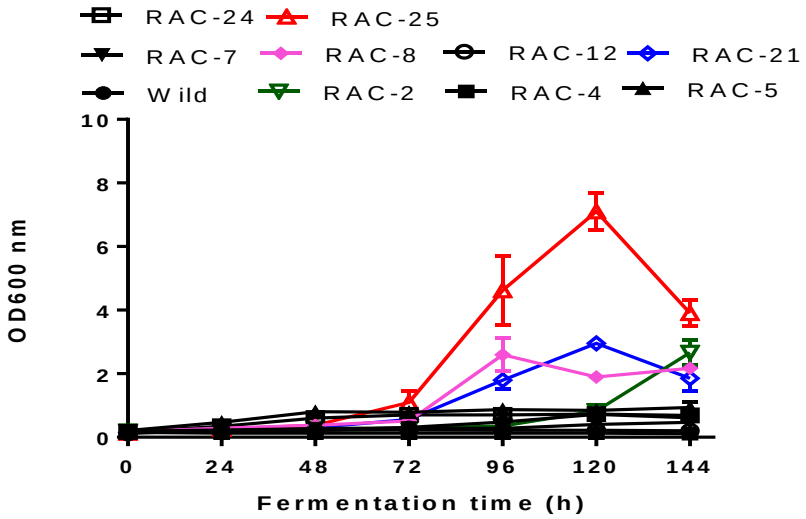


Figure 11. Comparison of microbial growth of nine mutants (RAC-2, RAC-4, RAC-5, RAC-7, RAC-8, RAC-12, RAC- 21, RAC-24 and RAC-25) and a wild type in MM supplemented with acetic acid (5 g/L) and HMF (0.04 g/L) during 144 h of batch fermentation. The experiments were carried out in duplicate.

Only four isolates (named RAC-2, RAC-8, RAC-21, and RAC-25) were able to reach an OD higher than 1.0. On the other hand, the wild type strain was not able to grow at all, confirming that ALE under HH promoted improved resistance toward acetic acid and HMF. Moreover, mutant RAC-25 revealed the best growth among the isolates; reaching a maximum OD_{600}nm of 7 in 120 h of fermentation. All mutants (RAC-2, RAC-8, RAC-21, and RAC- 25) were able to produce solvents despite the low acid production and the stressful environment imposed by the presence of acetic acid and HMF (Figure 12 and *Table 3S*, Supplementary Material).

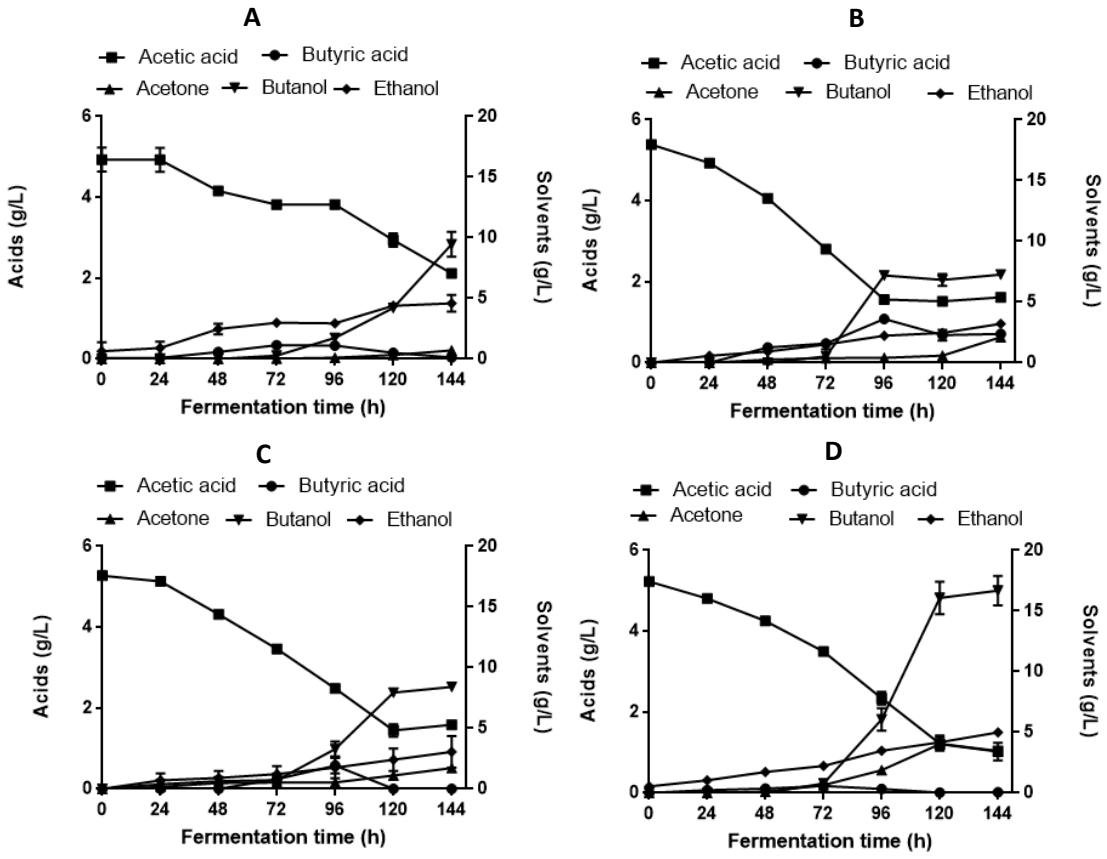


Figure 12. Profile of acid and solvent production of mutants RAC-2 (A), RAC-8 (B), RAC- 21 (C) and RAC-25 (D) during fermentation in mineral media containing acetic acid (5 g/L) and HMF (0.04 g/L).

It is well known that solventogenic *Clostridia* spp. have a typically biphasic metabolism, where during the first phase (acidogenic) acids are produced (acetic acid and butyric acid) concomitantly with microbial growth. Consequently, due to the low pH promoted by acid production, cells switch their metabolism to the next phase (solventogenic); in which the acids (acetic and butyric) are re-assimilated into solvents (acetone, butanol, and ethanol). Butanol production via this route is known as the "cold channel"[83]. Another alternative pathway is when butanol is directly produced from acetyl-coenzyme A (CoA) through butyryl-CoA, and is known as the "*hot channel*" [83]. In literature, it is reported that 15.1 g/L is the maximum titer of butanol produced by *C. saccharoperbutylacetonicum* under normal conditions without inhibitors (Fuel, 2017). The direct butanol forming hot channel has been described as playing a pivotal role in enhanced butanol production in comparison to cold channel [83]. Shinto et al. (2008) developed a model that showed *C. saccharoperbutylacetonicum* N1-4 has a robust metabolic network in the acid and solvent producing pathways [26]. The results obtained in our work suggest that the mutants (RAC-2,

RAC-8, RAC-21, and RAC-25) with high tolerance to inhibitors (acetic acid and HMF) tend to preferentially produce butanol from the "*hot channel*" instead of the "*cold channel*", since small amount of acids (acetic and butyric) were produced during fermentation (Figure 4). Our results are consistent with results obtained by Jin et al. (2015), where they observed a down regulation of the metabolic flux towards the acid formation branch ("*cold channel*"), and an up-regulation of the metabolic flux toward the ABE formation branches (hot channel); and consequently improved *C. acetobutylicum* fermentation of a non-detoxified wheat straw hydrolysate supplemented with sodium sulfite [116].

Mutant RAC-25 displayed excellent fermentative performance in MM containing acetic acid and HMF; consuming 84% of the sugars and producing 22.1 g/L of ABE ($Y_{ABE/S}$ = 0.42 g/g) (Table *3S*, supplementary material). Moreover, RAC-25 was able to achieve a cell density (OD_{600}nm of 7.0) similar to that observed for the wild type strain (OD_{600}nm of 9.14) in media without inhibitors (data not shown). Regarding butanol titer, mutant RAC-25 was able to produce 16.6 g/L of butanol ($Y_{but/s}$= 0.32 g/g); which is, to the best of our knowledge, the highest titer reported for batch cultures in a medium with a high concentration of acetic acid. In literature, 15.1 g/L is reported as the maximum titer of butanol produced by *C. saccharoperbutylacetonicum* under normal condition without inhibitors[35].

The remaining mutants (RAC-2, RAC-8, and RAC-21) were able to consume around 50% of the sugars and produce similar titers of butanol and ABE solvents. Nevertheless, mutant RAC-2 achieved the highest butanol ($Y_{but/s}$ = 0.34 g/g) and ABE yield ($Y_{ABE/S}$ = 0.50 g/g) despite consuming less sugar (46.7%) than the other mutants. Normally, both acetic and butyric acids are produced together with ATP generation in the acidogenic phase. These acids are then taken up for the production of butanol and ethanol, during the solventogenic phase, thus enabling an electron sink [83]. Another explanation for the high conversion yield observed in acetate containing media is the possible increased conversion of acetate to butanol to reduce its toxicity. Thus, a significant fraction of the solvents produced by the mutant RAC-2 can be formed from acetate. It is important to mention that an in-depth investigation, using metabolic modelling, for example, could be conducted to test this hypothesis, although no metabolic model for *C. saccharoperbutylacetonicum* is available until now.

In summary, our results revealed that the amount of butanol secreted by the mutant RAC-25 (16.6 g/L) in a batch fermentation exceeds previously reported limits for butanol tolerance for this bacteria [34] which leads us to conclude that the adaptive evolution brought genetic mutations that not only promoted tolerance to acetic acid and HMF but also increased the ABE production.

Genomic analysis of the evolved isolates

The results presented in the previous sections strongly support that the ALE strategy has generated mutants with higher growth capabilities as well as the best solvent production in the presence of inhibitors (acetic acid and HMF) compared to the parental strain. To provide more information about the different phenotype obtained throught ALE, we sequenced the genome of mutants (RAC-2, RAC-8, RAC-21, and RAC-25) and compared them to the wild type. Mutations were identified by whole-genome re-sequencing and each genome was compared with the parental strain (ID129676) in Genbank (NCBI). The results of the alignment process for each strain generated a mean mapping ratio of about 99.98% with high genome coverage (a least mean value of 123.7x) for each strain, which implied excellent quality for variant calling (*Table 4S*, Supplementary Material). The obtained mutations, related genes, and functional information are summarized in Table 9.

Table 9. Summary of mutations found in evolved strains.

Strain	Mutation	Type	Position	Gene	Function
RAC-2	Stop gained	SNP	Glu428*	CSPA_RS22950	Catalyzes the phosphorylation on incoming sugar substrates
	Deletion	Deletion	F171fs	CSPA_RS16265	Promote the attachment of RNA polymerase to specific initiation sites
	Missense	SNP	Leu3Ser	CSPA_RS14550	Uncharacterized protein
	Missense	SNP	Ile61Met	CSPA_RS17655	Oxidoreductase activity
RAC-8	Stop gained	SNP	Glu428*	CSPA_RS22950	Catalyzes the phosphorylation on incoming sugar substrate
	Deletion	Deletion	F171fs	CSPA_RS16265	Promote the attachment of RNA polymerase to specific initiation sites
	Missense	SNP	Leu3Ser	CSPA_RS14550	Uncharacterized protein
	Missense	SNP	Glu210Gly	CSPA_RS14135	Probably involved in glucitol uptake (carbohydrate transport)
	Missense	SNP	Gly30Ser	CSPA_RS00360	Protein involved in the pathway lipoprotein biosynthesis
RAC-21	Stop gained	SNP	Glu428*	CSPA_RS22950	Catalyzes the phosphorylation on incoming sugar substrates
	Deletion	Deletion	F171fs	CSPA_RS16265	Promote the attachment of RNA polymerase to specific initiation sites
	Missense	SNP	Leu3Ser	CSPA_RS14550	Uncharacterized protein
	Missense	SNP	Glu210Gly	CSPA_RS14135	Probably involved in glucitol uptake (carbohydrate transport)

	Missense	SNP	Gly30Ser	CSPA_RS00360	Protein involved in the pathway lipoprotein biosynthesis
RAC-25	Stop gained	SNP	Ser25*	CSPA_RS22795	Involved in the regulation of arabinose metabolism (repressor)
	Missense	SNP	Leu23Trp	CSPA_RS19575	Uncharacterized protein
	Missense	Complex	Lys271fs	CSPA_RS16260	Anti-sigma factor for Sigl regulation through direct interaction

Results indicate that some of the mutations were shared among the isolated mutants, while others were exclusively present in one of the mutants. To facilitate data analysis, we arranged the mutants into two groups: Mutants RAC-2, RAC-8 and RAC-21 who shared mutations in similar genes (CSPA_RS22950, CSPA_RS14550, CSPA_RS16265), while mutant RAC-25 presented mutations in different genes (CSPA_RS22795, CSPA_RS19575 and CSPA_RS16260).

The ability of biological systems to respond to various environmental or nutritional changes is directly correlated to biochemical and genetic networks [117]. In this sense, several genes are necessary for this complex process. Among them, we can mention the recognition by RNA polymerase associated with alternative sigma factors. We noted that two of the mutations found were present in genes related to sigma factors. Mutants RAC-2, RAC-8 and RAC-21 showed a deletion in gene CSPA_RS16265, which produces the RNA polymerase sigma factor I (sig I). Sigma factors are normally responsible for producing a multi-domain subunit of bacterial RNA polymerase, and therefore it plays an important role in transcriptional initiation [118]. Beyond that, this gene (*sigI*) is also involved in the regulation of cell wall metabolism in response to heat stress in *Bacillus* [119]. So far, this is the first work revealing a possible role of this specific sigma factor (*sigI*) in solventogenic *Clostridium* spp., since most of them have been reported in *Bacillus* spp. [120–122]. On the other hand, mutant RAC-25 showed a mutation (missense type) in the CSPA_RS16260 gene which produces the anti-sigma factor responsible for the downregulation of sigma factor I (*sigI*). Many works have described the involvement of transcriptional factors in stressful conditions, as well as strategies to enhance tolerance to many inhibitor compounds by manipulating these transcriptional factors [123–126]. Considering the mutations found in all the mutants, it was expected that RAC-2/RAC-8/RAC-21 showed a down regulation of *sigI*, since they presented a deletion in the gene responsible for *sigI* expression. On the other hand, regarding the mutant RAC-25, we expected a high expression of *sigI* due to a mutation in the *anti-sigI* gene, responsible for the *sigI* gene regulation.

Furthermore, we have also identified mutations in genes involved in membrane transport and the transcriptional regulators of carbohydrates. The mutant RAC-25 presented a mutation (stop gained) in the CSPA_RS22795 gene that belongs to the GntR transcriptional regulator family; which is a large group of proteins present in diverse bacteria and regulates various biological processes. This gene (CSPA_RS22795), named *araR* is responsible for the repression of genes related to arabinose metabolism and the pentose phosphate pathway in *Clostridium* spp [127]. In gram positive organisms the arabinose operon is negatively regulated by *araR*, binding to operator regions of the arabinose operon in the absence of arabinose. On the other hand, in the presence of arabinose the sugars bind to *araR* promoting conformational changes and preventing its binding to DNA [128]. It has been reported that concomitant downregulation of XylR and/or araR may improve mixed-sugar utilization in solventogenic *Clostridium* species [129]. In a study conducted by Zhang et al. (2012), the researchers used a comparative genomic approach to identify AraR-binding DNA motifs and reconstruct AraR regulons in nine different *Clostridium* spp. The results obtained indicated that the expression of genes related to the pentose phosphate pathway, like *tkt* (CAC1348), *tal* (CAC1347) and *ptk* (CAC1343), were up-regulated in the absence of arabinose in the mutant strain (*araR* inactivation) in comparison to wild type [127]. Their study corroborates with our results obtained from mutant RAC-25, which indicate that the mutation in gene *araR* could de-repress genes involved in xylose metabolism and improve sugar uptake (Figure 11, *Table 3S and Figure 3S*, Supplementary Material).

It has been shown that the inactivation of the XylR transcriptional repressor has been associated with increased utilization of xylose as the main substrate in *C. beijerinkii* and *C. acetobutylicum* [91,130]. A study conducted by Xiao et al. (2017) evaluated a point mutation in DNA dependent RNA polymerase (*ropB*) regarding osmotolerance and succinic acid production in *E. coli*. The authors showed that the mutation rendered *E. coli* resistant to osmotic stress, probably due to improved cell growth and viability via enhanced sugar uptake under stress conditions, and activated a potential "pre-defense" mechanism under non-stressed conditions [131].

Another mutation (stop gained) shared by mutants RAC-2, RAC-8 and RAC-21 is present in gene CSPA_RS22950 (*glcB*), which encodes the glucose specific EIICBA protein component of the PTS (phosphotransferase system) system. The PTS system carries out both catalytic and regulatory functions in microbial cells. It plays an important role in transport mechanism of carbohydrate substrate, catalyzing both the accumulation and chemical conversion (phosphorylation) [132]. Since it has an important role in sugar uptake, we

expected the mutation in CSPA_RS22950 (stop gained) would impact negatively the microbial growth and butanol production. Indeed, this mutation showed a negative effect on microbial cells, impacting the substrate uptake and energy metabolism in MM with (Table 3) and without inhibitors (Figure *3S*, Supplementary Material). As mentioned before, this could be a strategy of cells, similar to catabolic repression, to consume the acetate present in media to avoid the deleterious effect caused by this acid at high concentrations. However, it is important to point out that there is a lack of knowledge describing a possible strategy to overcome hostile acidic conditions. Therefore, additional studies are required to deeply investigate this hypothesis of carbon catabolite repression (CCR) to promote acetate consumption.

Gene expression via RT-qPCR of the evolved isolates

Based on the results presented above, we hypothesized that in the first group (RAC-2, RAC-8 and RAC-21) sigma factor expression should be decreased, whereas in the second group (RAC-25) its expression should be increased when compared to the parental strain. To verify our hypotheses, we evaluated the expression level of sigma factor I and other genes related to stress conditions in two mutants from each group (RAC-21 and RAC-25), in comparison to the wild type strain. The genes investigated were: *sigI* (CSPA_RS16265), *proA* (CSPA_RS00190), *groL* (CSPA_RS02180) and *atpD* (CSPA_RS03060) (Figure 13).

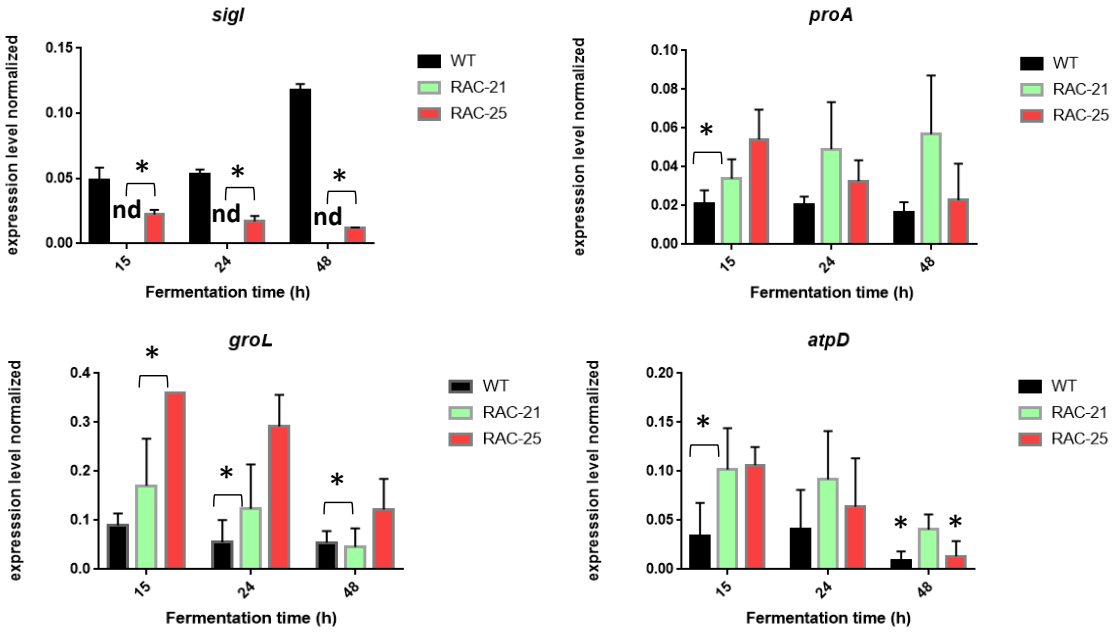


Figure 13. Real-time PCR of genes involved in stress conditions for the wild type (WT) and mutants RAC-21 and RC-25 obtained by ALE. The genes investigated are: *sigI* (Csps_c33520), *proA* (Cspa_c00390), *groL* (Cspa_c04430) and *atpD* (Cspa_c06260). ND: not detected.

The results indicated that the expression of *sigI* was significantly different ($p < 0.005$) in the mutants studied (RAC-21 and RAC-25) compared to the wild type (WT) in all tested cultivation times (15, 24 and 48 h) (Figure 13). Moreover, the mutant RAC-21 did not express the *sigI*, as expected, due to the deletion of this gene, confirming the results obtained in the genome sequencing. On the other hand, the mutant RAC-25 surprisingly revealed a lower expression of the *sigI* gene in comparison to the wild type (WT). Down-regulation of *sigI* might be explained by the fact that the mutation in the anti-sigma factor can affect the mechanism responsible for "switching-off" the sig I protein; promoting a phenotype similar to the other mutants (RAC-2, RAC-8 and RAC-21). In the work performed by Minty et al. (2010), experimental evolution was applied to obtain *E. coli* mutants tolerant to exogenous isobutanol. Their results showed that many isobutanol tolerant strains presented a reduced activity in RpoS (sigma factor), probably related to a mutation in *hfq* or a*cr*AB. They concluded that the mechanism for adaptation to isobutanol was based on cell envelope remodeling and stress response attenuation [133]. In another work, Riordan et al. (2010) showed that the inactivation of alternative sigma factor 54 (*rpoN*) affected the expression of stress resistance genes, most notably the gad genes required for GDAR (glutame-dependent acid resistance); promoting an increase in acid resistance in the mutant strain [134]. Our results with *sigI* suggest that the low expression of *sigI* can promote an improvement in tolerance of *C. saccharoperbutylacetoncium* towards acetic acid and HMF. However, it is important to note that until now, no other work has described which genes are regulated by *sigI* (CSPA_RS16265) in solventogenic *Clostridium* spp.

Beyond *sigI*, we also evaluated the expression of other genes involved in stressful conditions (proA, atpD and grol). The expression of the gamma-glutamyl phosphate reductase (*proA*) gene, that is involved in L-proline biosynthesis [135], was also evaluated (Figure 13). The results indicated a higher expression level of *proA* throughout cultivation in both mutants (RAC-21 and RAC-25) in comparison to the wild type. However, statistical analysis showed only differences between RAC-25 and WT at 15 hours of cultivation. The results suggest that high expression of *proA* could be related to the improved tolerance to lignocellulosic inhibitors, in this case to acetic acid and HMF. Our data corroborates with results obtained by Liao et al. (2018), who showed that overexpression of some genes in (*proA, proB, and proC*) *C. acetobutylicum* to enhance proline biosynthesis promoted an excellent ability to withstand inhibitors (formic acid, ferulic acid, *p*-coumaric acid and syringaldehyde); and efficiently fermented undetoxified hydrolysates from different raw materials (soybean straw, rice straw, and corn straw) [136].

The *grol* gene which produces the 60 kDa chaperonin was also evaluated. This gene is responsible for preventing misfolding and promoting the refolding; and proper assembly of unfolded polypeptides generated under stress conditions. Our results indicate higher expression of *grol* in mutants (RAC-21 and RAC-25) at 15 and 24 h of cultivation (Figure 13). Statistical analysis only showed significant differences between mutants and the wild type at 15 h, and between mutant RAC-25 and WT at 24 and 48 h. In a study conducted by Tomas et al. (2003), it was observed that the overexpression of the *groELS* gene in C. *acetobutylicum* promoted an increase of butanol tolerance and solvent production [137]. In another study the researchers constructed a recombinant strain of *C. beijerinkiii* NCBI 8052 to overexpress *groES* and *groEL* and observed a higher solvent production, even under ferulic acid stressed conditions; providing a good candidate strain for biomass hydrolysate fermentation [138].

Finally, to investigate the acid tolerance of mutants, we evaluated the expression of H^+ATPase (ATP synthase); since the response to organic acids, cells have demonstrated an increase in membrane H^+ATPase activity through dissipation of plasma membrane potential induced by the weak acids[139]. It is known that uncharged weak acids can difuse freely across plasmatic membrane. Due to a more neutral intracellular pH, charged anions and protons are retained within cell, and cytoplasmic protons are expelled by membrane bound H^+ATPase [139,140]. Beyond disrupting internal pH homeostasis, weak acids can also affect lipid organization and function of cellular membranes [139]. The data obtained regarding *ATP synthase subunit beta* expression showed a higher expression in mutants RAC-25 and RAC-21 at 15 and 24 h of cultivations in comparison to the wild strain; presenting statistically significant differences only at 15 h between RAC-25 and WT (Figure 13). At the end of cultivation (48 h), all the strains (mutants and WT) showed a decreased expression of this gene. In recent work, Mamata et al. (2018) applied adaptive laboratory evolution to improve *Lactobacillus delbriecki* FMI performance at low pH (4.5) and showed a 1.80-fold increase in lactic acid production compared to the parental strain. Moreover, the evolved strain exhibited a higher H^+ATPase activity, as well as a higher *H^+ATPase* gene expression compared to the parent strain [141]. Guan et al. (2018) performed comparative genomics and transcriptomics analysis in an acid-tolerant strain of *Propionibacterium acidipropionic* to understand the microbial response of cells to acid stress during fermentation. The results showed that genes involved in ATP synthesis were found to differ in copy numbers between the two strains (evolved and parental strain). Thus, they concluded that several transporters, membrane proteins, and the ATP synthase delta chain contributed to phenotype differences between the wild type strain and an acid-resistant mutant [142]. The result confirms our data, supporting

that an up-regulation of both ATP synthases (beta and delta subunits) may contribute to the enhanced acid tolerance displayed by RAC-21 and RAC-25 mutants.

Scanning electron microscopy (SEM)

In the adaptive laboratory evolution strategy, we observed some cellular morphological changes during cultivations under routine light microscopy observation (data not shown). Therefore, we decided to investigate these changes in morphology using SEM. Images of three mutants (RAC-2, RAC-21, and RAC-25) and the wild type in the mid-exponential phase of cultivation (15 h) were obtained by SEM (Figure 14).

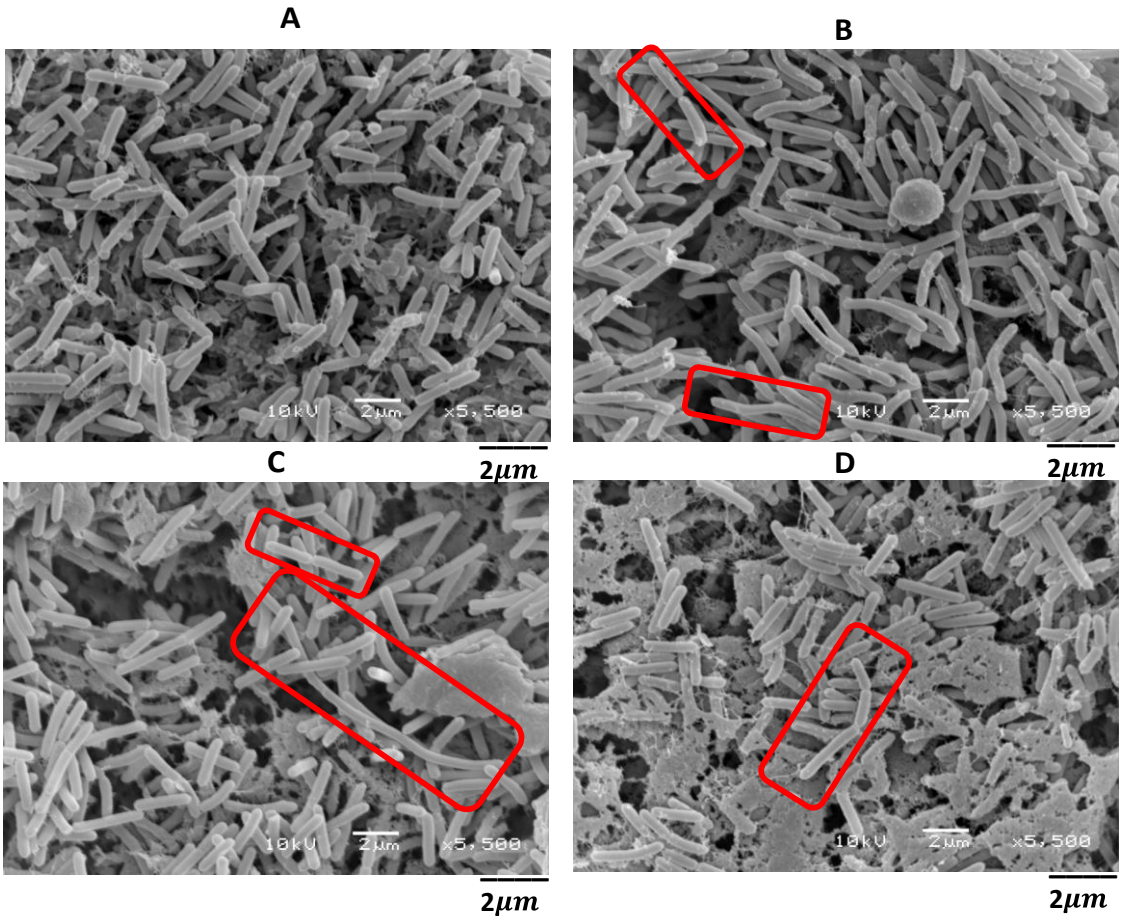


Figure 14. Scanning electron microscopy of *C. sacharoperbutylacetonicum* (DSMZ 14923) cultivated in RCM (without inhibitors) at 24 h: A) Wild type, B) RAC-2, C) RAC-21 and D) RAC-25.

The images revealed the differences between wild type (Figure 14A) and evolved strains (Figure 14 B, C, and D). It is shown that the mutants were much more elongated (almost 2 twofold) in length in comparison to the wild type (control experiment). We believe that these

changes can be related to the mutation found in *sig I* and *anti-sig I* which directly affect *sigI* expression, as previously observed in Figure 13. Alterations in cell morphology have been described as a visible indicator of bacterial strategies to tackle different environmental stress conditions [143]. In recent work, Zhang et al. (2017) performed a comparative transcriptome analysis of a *C. beijerinkii* degenerated strain and the wild type 8052 strain. They found that morphological and physiological changes in the degenerated strain DG-8052 were related to disturbed expression of sigma factors; affecting aspects of sugar transport and metabolism, sporulation, chemotaxis and solventogenic pathways [144].

Conclusions

In this work, four robust strains of *C. saccharoperbutylacetonicum* able to withstand a high concentration of acetic acid and HMF were successfully obtained through ALE. The genome analysis indicated that a down-regulation of *sigI* can be directly involved in the improved tolerance of those strains. Moreover, the genes involved in membrane transport and metabolism of carbohydrates seem to be linked to a cellular strategy for adaptation to the challenging environment promoted by inhibitors. Our results bring important information about genes directly related to tolerance mechanism of cells, suggesting interesting targets for future metabolic engineering to obtain robust strains of *C. saccharoperbutylacetonicum*.

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5. GENERAL DISCUSSION

This work highlighted important results regarding the potential tolerance mechanisms of *C. saccharoperbutylacetonicum* to withstand the main inhibitors present in sugarcane-based hemicellulosic hydrolysate (HH) for butanol production. For this purpose, we applied adaptive laboratory evolution (ALE) to obtain evolved strains that tolerate the main inhibitors of *Clostridium sacharoperbutylacetonicum* (acetic acid and HMF) present in substrates. Selected

evolved mutants (RAC- 2, RAC-8, RAC-21 and RAC-25) were thoroughly characterized; in terms of their physiology and fermentative performance, and their genomes were sequenced in order to gather information about mutations that could be associated with the improved robustness of the evolved phenotypes.

In the first chapter, we studied four clostridium strains (*C. acetobutylicum*, *C, beijerinkii*, *C. sacharobutylicum and C. saccharoperbutylacetonicum*) reported as butanol producers [80]. The focus of this first part was to select the best strain regarding xylose consumption, butanol titer and yield; and then move forward to applying ALE to increase robustness towards HH. The strain selected as the best butanol producer was then comprehensively studied in regard to the effects of sugar concentration, kinetic parameters and butanol tolerance. The results showed that among the strains studied (*C. acetobutylicum*, *C. beijerinkki*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*), *C. saccharoperbutylacetonicum* stood out as the highest butanol producer; achieving a butanol yield of 0.24 g/g in xylose and 0.31 g/g in glucose (in cultivations with 30 g/L of initial sugar concentrations). One of the issues that we faced during the development of this first part of the work was the fact that this strain (*C. saccharoperbylacetonicum*) was marginally studied, and not much data is published in the literature to compare with our own data.

In the second chapter, we achieved robust strains of *C. sacchaperbutylacetonicum*; able to tolerate the main inhibitors present in HH. By applying ALE for 130 generations in a repetitive batch mode, we obtained an evolved population of *C. saccharoperbutylacetonicum* (referred to as EP-40) with a high capacity of tolerance against the main inhibitor compounds in HH, compared to the parental strain. In total, nine isolated colonies from EP-40 were selected to be investigated further under the presence of HH-derived inhibitors. Due to difficulties in evaluating the evolved mutants together with the parental strain in HH-based cultivation media (40% of HH), we evaluated the strains in media supplemented with selected inhibitory compounds; namely acetic acid and HMF. In a previous experiment, we evaluated the effects of fourteen (14) inhibitors present in HH on the microbial growth of *C. saccharoperbutylacetonicum.* The results indicated that between all the tested compounds, only acetic acid and HMF negatively impacted microbial growth in the range of concentrations studied. It is important to note that only one published work in literature evaluated the impacts of lignocelulosic inhibitors on *C. saccharoperbutylacetonicum* metabolism [35]. In this work the researchers observed that *p*-coumaric acid, ferulic acid and syringaldehyde were potent phenolic inhibitors; with *p*-coumaric acid being the most toxic for microbial cells. Moreover, HMF and furfural were inhibitory but not as toxic as the phenolic compounds. It is important to

highlight that the concentrations of inhibitors examined in this work were higher (0.2 g/L – 1 g/L for phenolic compounds, and 1 g/L – 4 g/L for HFM and furfural) than those used in our work. These results gave valuable information about the impact of the main inhibitors present in lignocellulosic biomasses on *C. saccharpebutylacetonicum* growth. However, it is important to note that the researchers nor did other authors test the effects of acetic acid on this microorganism. The literature has also not yet reported any work that evaluated the effects of acetic acid in *C. saccharoperbutylacetonicum*, and we believe our results could shed new light on this potent inhibitory compound on ABE fermentation using this strain.

As a continuation, we deemed it necessary to evaluate the fermentative performance of isolated mutants obtained from the ALE strategy. Therefore, the evaluation of the evolved mutants was performed using mineral media (MM) containing acetic acid and HMF, at the same concentrations found in 40% of HH. As a result, we were able to observe differences in microbial performance of the strains; where four evolved strains (named RAC-2, RAC-8, RAC-21 and RAC-25) were able to grow efficiently, while the wild type did not. One hypothesis to explain the differences between cultivation in MM supplemented with HH (40%) and MM supplemented with AA and HMF; is that the inhibitors present in HH could be degraded due to longer storage time (2 years) in the cold (4 °C). It is important to note that despite many works in the literature describing the adoption of the ALE strategy to increase inhibitor tolerance in different microbial cells, there is a lack of work with solventogenic *Clostridium* spp.; as can be observed in table 10.

Table 10. Adaptive laboratory evolution in different microbial cells for increased tolerance towards lignocellulosic derived inhibitors.

Microorganism	Selective Pressure	Cultivation Mode	Media	Selection time	References
C. saccharoperbutylacetonicum	Lignocellulose derived inhibitors	Repetitive batch mode	Sugarcane Bagasse hydrolysate	130 generations	This study
Rhodosporidium toruloides	Lignocellulose derived inhibitors	Repetitive batch mode	Sugarcane Bagasse hydrolysate	nd	[69]
C. thermocellum	Lignocellulose derived inhibitors	Repetitive batch mode	Populus hydrolysate	117 transfers	[145]
Saccharomyces cerevisiae	Lignocellulose derived inhibitors	Batch and Continuous mode	Minimal medium containing inhibitors and spruce hydrolysate	526 generations	[68]
Corynebacterium glutamicum	Lignocellulose derived inhibitors	Repetitive batch mode	Corn stover hydrolysate	130 transfers	[70]
Spathaspora passalidarum	High concentration of acetic acid	UV irradiation and continuous cultivation	Synthetic media with increasing acetic acid concentration	380 generation	[146]
Saccharomyces cerevisiae	High concentration of acetic acid	Repetitive batch mode	Synthetic media with increasing acetic acid concentration	50 transfers	[104]
Aureobasidium pullulans	Acetic acid present in rice hull hydrolysate	Repetitive batch mode	Rice hull hydrolysate	20 cycles	[147]
Zymomonas mobilis	Furfural and acetic acid present in Lignocellulosic hydrolysate	Repetitive batch mode	RM supplemented with furfural and acetic acid	3 rounds	[148]

The four selected mutants were genome sequenced using CTBE/CNPEM facilities; to identify possible mutations that could explain the acquired tolerance during ALE experiments. The bioinformatics task and data analysis were carried out at the University of Minho (Portugal) in collaboration with Professor Isabel Rocha, for six months in an internship funded by Capes (88881.135385/2016-01). Genome analysis allowed us to identify some mutation in genes related to carbohydrate metabolism and stress-factors (sigma factor I). When we sequenced the genome of evolved cells, we were expecting to find a substantial number of mutations, as a complex strategy of cells to adapt to the inhibitors present in cultivation media. Normally, ALE approaches usually generate robust tolerant strains to specific inhibitors presenting many mutations in the genome [130,149]. In the present work, ALE generated a low number of mutations in the evolved strains, which facilitated our task to elucidate the molecular basis regarding the inhibitor tolerance mechanism of *C. saccharoperbutylacetonicum*.

One important observation was that, apparently, evolved strains used different strategies regarding sugar uptake/metabolism, to overcome the challenges imposed by the inhibitors. This is confirmed by the fact that some mutants (RAC-2, RAC-8 and RAC-21) were not able to display good fermentative performances in media (with or without inhibitors), since the cells did not efficiently consume sugars present in media. On the other hand, mutant RAC-25 showed an excellent fermentative performance under both media conditions (with and without inhibitors); efficiently consuming sugars and producing the highest butanol titer (16.7 g/L) in media containing acetic acid (5g/L) and HMF (0.4g/L) currently reported.

The previous results regarding sugar uptake and fermentative performance are in accordance to the mutations found in the PTS gene system (RAC-2, RAC-8 and RAC-21) and *araR* gene (RAC-25). The first gene has a negative impact on fermentative performance, whereas the second has a positive impact in acidic media conditions, containing 5 g/L of AA. Despite the different mutations in the sigma factor I gene (Cspa_c33520) and in the anti-sigma factor I gene (Cspa_33510) in evolved strains, RT-qPCR analysis showed that both promoted a down regulation of the sigma factor transcript in all mutants (RAC-2, RAC-8, RAC-21 and RAC-25); suggesting that the low expression of *sigI* can promote an improvement in tolerance of *C. saccharoperbutylacetoncium* towards acetic acid and HMF. Finally, the scanning electron microscopy analysis allowed us to confirm morphological changes in the

evolved strain phenotypes; with the cells being significantly more elongated in comparison to the wild type.

In summary, we have shown a set of genetic adaptations in cells to tolerate acetic acid and HMF present in HH. Our results bring important information about genes directly related to tolerance mechanisms in *C. saccharoperbutylacetonicum*. We suggest that *sigI* and *araR* genes may be interesting targets to obtain robust strains with high tolerance to inhibitors derived from biomass; and with the potential to produce higher titers of butanol.

6. CONCLUSION

Based on the results obtained we concluded that:

- Among the wild type strains studied (*C. acetobutylicum DSM6228, C. beijerinkii DSM6422, C. saccharobutylicum DSM13864* and *C. saccharoperbutylacetonicum DSM14923*), *C. saccharoperbutylacetonicum DSMZ 14923* displayed the best fermentative performance for butanol production; and 50 g/L was the most suitable initial sugar concentration to obtain the maximum butanol titer and yield;

- Wild-type *C. saccharoperbutylacetonicum* is able to tolerate a maximum butanol concentration of around 12 g/L;

- Evaluation of fourteen different inhibitor compounds usually present in HH on the growth of *C. saccharoperbutylacetonicum* indicated that acetic acid and HMF were the only compounds that significantly reduced this parameter, in the range of concentrations evaluated;

- The adaptive laboratory evolution strategy on hemicellulosic hydrolysate (HH) based medium generated an evolved population after around 130 generations, from which four isolated mutants (RAC-2, RAC-8, RAC-21 and RAC25) were able to grow in the presence of acetic acid (5 g/L) and HMF (0.04 g/L); a condition that completely abolished growth of the wild type strain;

- Among the isolated mutants, evolved strain RCA-25 displayed the best fermentative performance in the presence of inhibitor compounds, producing 16.7 g/L of butanol and consuming 84% of the sugars provided during 144 h of batch fermentation;

- Genome sequencing identified some mutations in genes related to stress, such as sigma factors and anti-sigma factors, and genes related to sugar uptake/metabolism; which could be linked to the improved phenotype;

- The mutants RAC-2, RAC-8 and RAC-21 showed a mutation in the gene Cspa_c47240, that promoted low sugar uptake efficiency, suggesting some kind of carbon catabolite repression (CCR); a strategy to consume acetate in order to detoxify the fermentation media. On the other hand, the RAC-25 strain presented a mutation in the Cspa_46930 gene that induced better sugar uptake and metabolism of sugars present in media.

- Despite the different mutations found in sigma and anti-sigma factor I in all mutants (RAC-2, RAC-8, RAC-21 and RAC-25), the RT-qPCR revealed a similar effect on *sigI* expression, suggesting that a down regulation of this gene can be directly involved in *C. saccharoperbutylacetonicum* inhibitor tolerance.

- Our results bring important information about genes directly related to the tolerance mechanism of *C. saccharoperbutylacetonicum*, suggesting interesting targets for metabolic engineering to obtain robust strains with high tolerance to lignocellulosic derived inhibitors compounds.

7. SUGGESTION FOR FUTURE WORK

- As we were able to produce mutants with a high capacity to withstand AA and HMF, it would be interesting to evaluate the effects of other inhibitors present in HH on the microbial growth of RAC-25, as this was the best butanol producer. Moreover, it would be interesting to continue the ALE in higher concentrations of HH, to increase the robustness of the strain and then further evaluate the fermentative performance in different hydrolysates obtained through other pre-treatments.

- It would be valuable to evaluate the butanol tolerance of RAC-25, since we observed a high butanol production as a consequence of the mutation generated by ALE. Moreover, we could further investigate the changes in cell metabolism using FBA (Flux balance analysis) and other “-omics” tools such as transcriptomics and proteomics.

- Use ALE to improve tolerance to solvents in the media of the *C. saccharoperbutylacetonicum* RAC-25 strain;

- Since the mutants RAC-2, RAC 8 and RAC-21 were not able to properly consume the sugars present in media (with or without inhibitors), it would be interesting to evaluate if the cells developed a capacity to efficiently consume the acetate present in media, as a sole substrate; to develop a strategy to quickly consume this compound in order to detoxify media and survive.

- Finally, since we were able to identify mutations in the evolved cells with high AA and HMF tolerance capacity, it would be extremely important to use the metabolic engineering approach to make genetic modifications in WT strains, mainly in *sigI* and *araR*; and evaluate the tolerance capacity of the genetically modified strains obtained, in order to confirm that these genes mentioned are directly responsible for inhibitor tolerance in *C. saccharoperbutylacetonicum*.

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9. SUPLEMENTARY MATERIAL

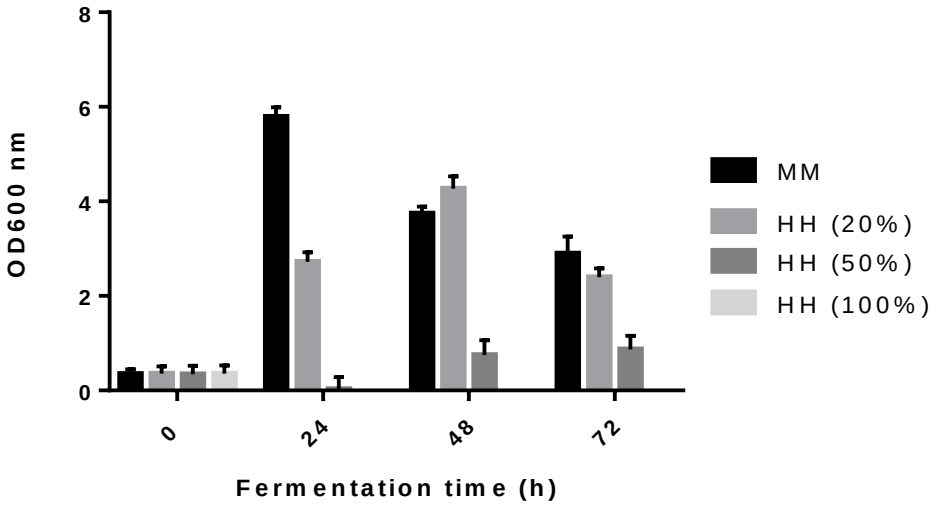

Figure 1S. Microbial growth of a wild strain of *C. saccharoperbutylacetonicum* cultivated in mineral media containing HH (20%, 50%, and 100%). Mineral media (MM) without inhibitors was used as a positive control. The experiment was carried out in duplicate.

Table 1S. Inhibitory compounds concentrations at each level of the Plackett-Burman screening design.

Compounds	High level +1 (g/L)	Central 0 (g/L)	Low level -1 (g/L)
HMF	0.3	0.17	0.03
Furfural	0.01	0.006	0.001
Acetic acid	2.6	1.43	0.26
Syringaldehyde	0.2	0.11	0.02
Glucuronic acid	0.2	0.11	0.02
p-coumaric acid	0.16	0.09	0.016
p-Hydroxybenzoic acid	0.2	0.11	0.02
Vanillic acid	0.05	0.03	0.005
Levulinic acid	0.2	0.11	0.02
Formic acid	0.3	0.17	0.03
Ferulic acid	0.15	0.08	0.015
Phenylacetic acid	0.2	0.11	0.02
Vanilin	0.15	0.08	0.015
Syringic acid	0.07	0.04	0.007

Table 2S. Plackett-Burman screening design to evaluate the effect of fourteen compounds commonly found in hemicellulosic hydrolysates on *C. sacharoperbutylactonicum* growth. Dependent variable is the optical density at 600nm at 24 h of fermentation. The experiment was designed using Minitab 14.5 (Minitab LLC, USA).

Run	HMF	FUR	AAC	SER	GLUC	PCOU	BZA	VAA	LEA	FOA	FEA	PAC	VAN	SEA	OD_{600nm}
1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	2.3
2	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	0.5
3	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1.0
4	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	1.9
5	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	0.3
6	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	3.2
7	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	3.1
8	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	0.3
9	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	0.2
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.7
11	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	0.4
12	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	2.7
13	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	3.1
14	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	2.7
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.0
16	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	3.1
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.9
18	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	2.2
19	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	4.9
20	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	0.3
21	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**	**5.0**
22	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	3.0
23	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	3.0

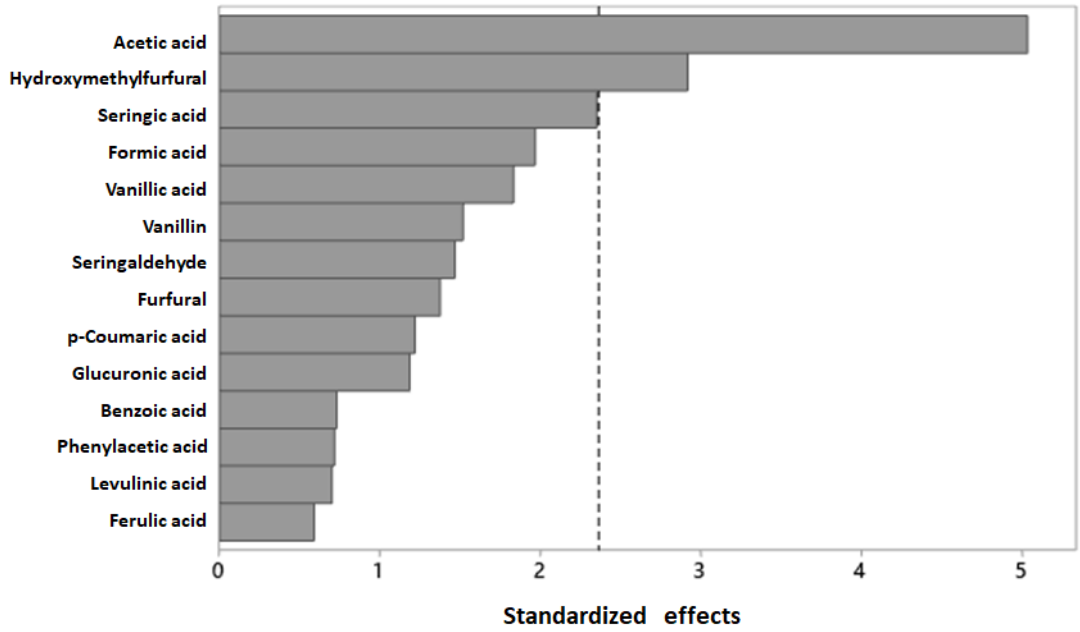

Figure 2S. Pareto chart to evaluate the effect of fourteen compounds on *C. saccharoperbutylacetonicum* growth at 24 h of fermentation based on the Placket-Burman design presented in Table 2S. Bars crossing the dashed line indicate statistically significant factors at 95% of confidence level. Data was analyzed using Minitab 14.5 (Minitab LLC, USA).

Table 3S. Fermentative performance of mutants in 144 h of batch fermentation in MM supplemented with acetic acid (5g/L) and HMF (0.04 g/L).

Mutant	**OD_{600nm}**	**Titer (g/L)**		***Yield[(c)] (g/g)**		**Productivity (g/L.h)**		**Residual sugar (%)**
Media supplemented with acetic acid and HMF								
		Butanol	ABE	Butanol	ABE	Butanol	ABE	Xylose
RAC-2*	2.66	9.45	14.76	0.34	0.50	0.065	0.10	53.3
RAC-8*	2.59	7.16	13.94	0.24	0.42	0.049	0.096	50
RAC-21*	2.95	8.40	14.57	0.26	0.41	0.058	0.10	47
RAC-25*	7.01	16.66	22.69	0.32	0.42	0.11	0.15	16

* Yield calculated based on total sugars consumed during fermentation. For this experiment we used 60 g/L of total sugar.

Table 4S. Summary of read alignments of the evolved strains.

	RAC-2	**RAC-08**	**RAC-21**	**RAC-25**	***Wild Type**
Total # of reads	5977975	7808994	5971466	7637405	12097727
Properly mapped reads	5966842	7804261	5966411	7629130	12084835
Mapping ratio	99.92%	99.94%	99.92%	99.89%	99.89%
Mean base coverage	123.7	166.0	126.0	162.1	257.6
Total reference bases			6666445		

*Wild Type: *C. saccharoperbutylacetonicum* (DSMZ 14923)

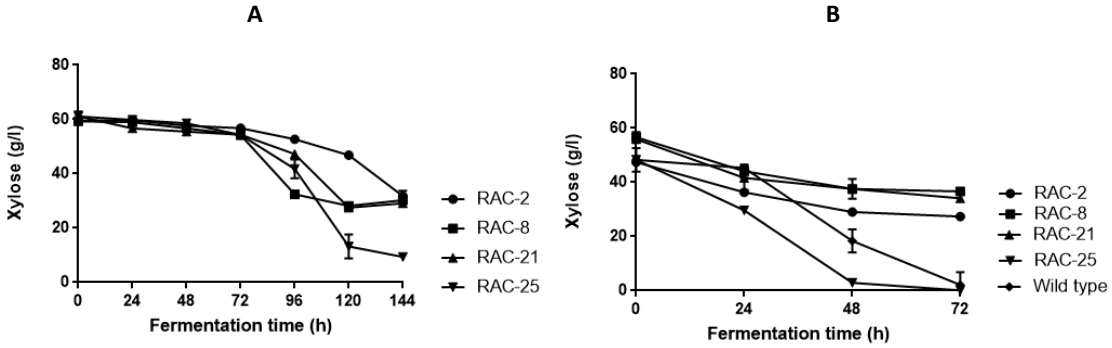

Figure 3S. Profile of sugar consumption of mutants (RAC-2, RAC 8, RAC-21 and RAC 25) during cultivation in media: (A) MM with acetic acid (5g/L) and HMF (0.04 g/L), (B) MM without inhibitors.