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Research paper

Towards enhanced *n*-butanol production from sugarcane bagasse hemicellulosic hydrolysate: Strain screening, and the effects of sugar concentration and butanol tolerance

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Butanol tolerance

A B S T R A C T

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.

1. 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 [1]. To overcome such limitations, recent studies have focused on the optimization of the ABE fermentation process and strain development using several metabolic engineering strategies [2]. 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 [3].

The economics of butanol production can certainly benefit from existing sugarcane ethanol mills in countries such as Brazil, Colombia,

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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 [5]. 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. [7] 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 as sole carbon source. 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.

2. Material and methods

2.1. 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.

2.2. 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) 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.

2.3. 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 %) [11]. 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.

2.4. 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.

2.5. 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 [12]. RT in each sampling time (*t*) is given by Eq. (1), in which control refers to a fermentation without butanol addition.

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

2.6. 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 Brazilian Bioethanol Science and Technology Laboratory (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 (Fig. 1). The hydrolysis 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 °C and 200 rpm for 30 min. These conditions were previously determined [11] 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. All the procedures were carried out under sterile conditions in a laminar flow hood. The composition of the hemicellulosic hydrolysate is presented in *Table* 1.

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 1. 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.

Table 1
Composition of the sugarcane bagasse hemicellulosic hydrolysate obtained in 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

2.7. 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 °C; 1:8 vol 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

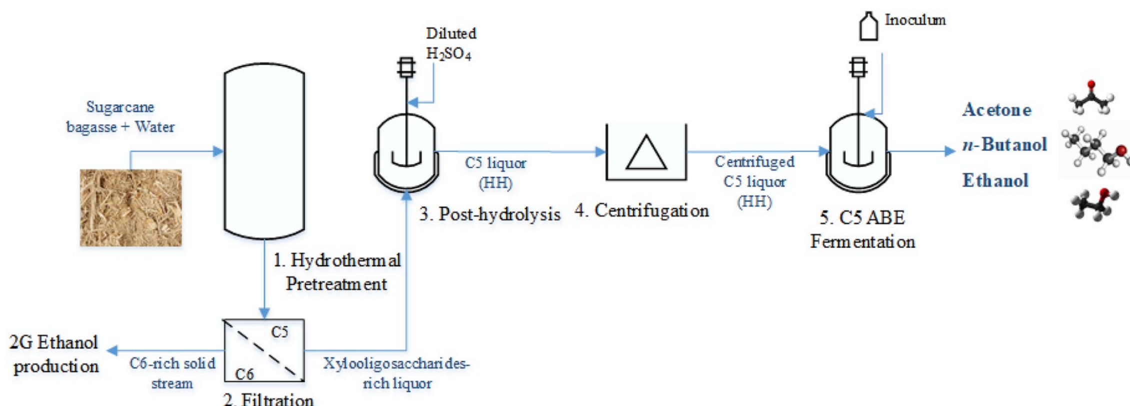

Fig. 1. Schematic diagram of the production of sugarcane bagasse hemicellulosic hydrolysate (HH) and its use for ABE production.

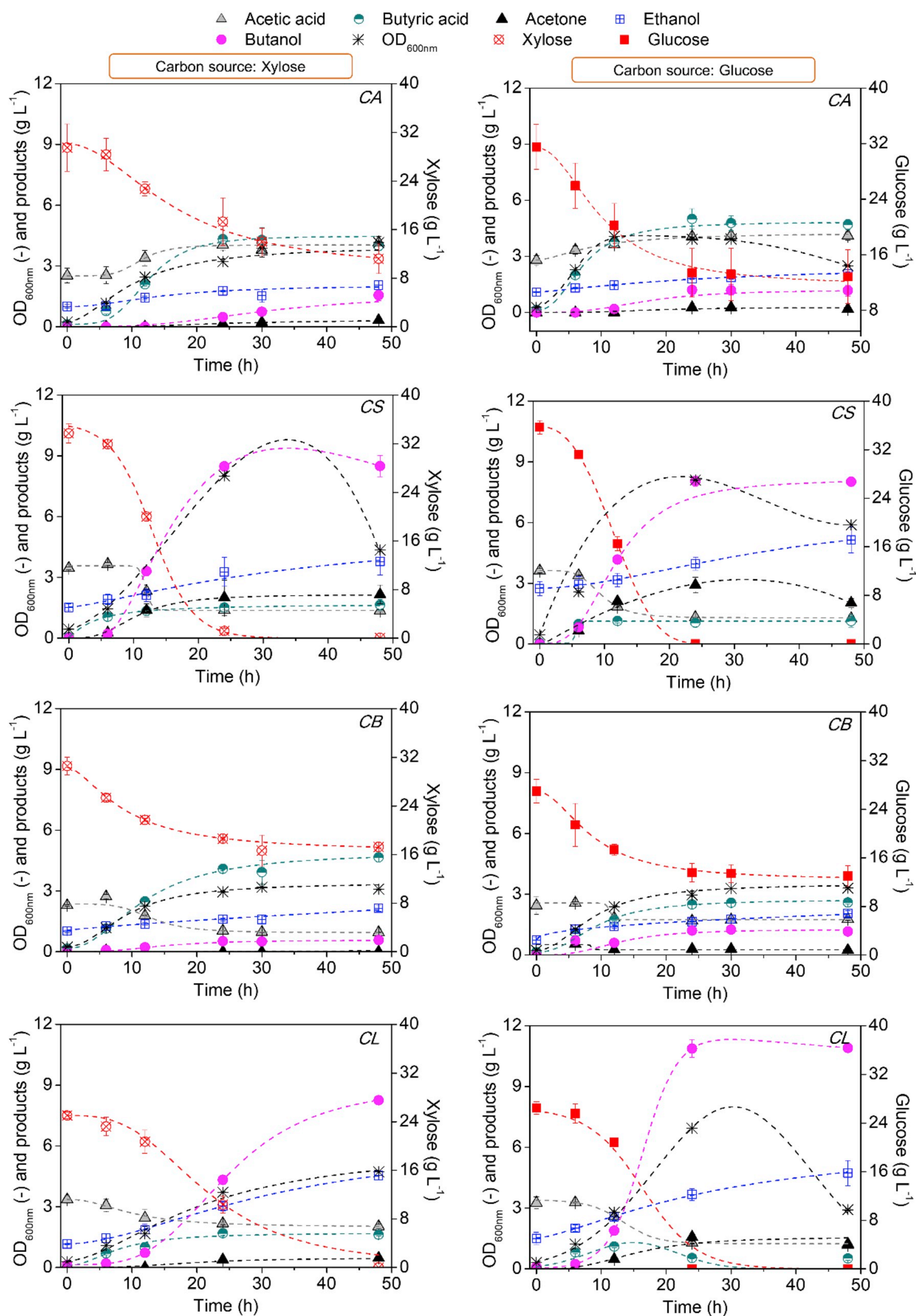

Fig. 2. Production of ABE and acids, cell growth, and sugar consumption in ABE fermentations to screen the *Clostridium* strains. Xylose fermentation on the left column and glucose fermentation on the right column. CA: *C. acetobutylicum* DSM 622, CB: *C. beijerinckii* DSM 6422, CS: *C. saccharobutylicum* DSM 13864, and CL: *C. saccharoperbutylacetonicum* DSM 14923. Dashed lines represent a general tendency.

Table 2
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 3
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% Glu)	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

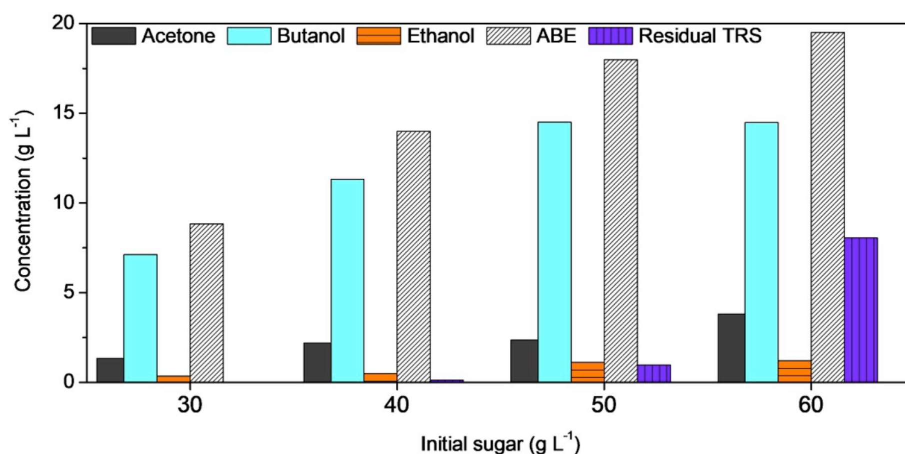

Fig. 3. Effect of initial sugar concentration on production of ABE and sugar consumption by *C. saccharoperbutylacetonicum* DSM 14923. Synthetic fermentation medium contained mixed sugars (97% xylose and 7% glucose). Fermentation time was 48 h.

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 (Eqs. (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.

$$DCW_{growth} = 0.4065 \times OD_{600nm} \quad (r2 = 0.98) \tag{2}$$

$$DCW_{death} = 0.325 \times (OD_{600nm} + 3.20) \quad (r2 = 0.99) \tag{3}$$

3. Results and discussion

3.1. 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}] (Fig. 2). 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 2). 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

Table 4
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	50.0	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 4923	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	[22]
	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	[23]
C. beijerinckii NCIMB8052	Xylose-rich medium	–	–	5.0	0.28	24.4	–	[24]
C. acetobutylicum DSM 1731	Xylose	5	0.25	3.6	0.18	20.1 (from 50 g L^{-1})	0.72	[25]
	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	[26]
	Xylose (80% xyl) + starchy slurry	4.84	0.22	3.08	0.14	22.0 (from 50 g L^{-1})	0.63	

(-) not reported.

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. [13].

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}) (Fig. 2). As a result, yields were lower than 0.1 g ABE g^{-1} (Table 2). 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 [14], 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" [15]. This phenomenon was observed in other studies on ABE fermentation by *C. acetobutylicum* ATCC 824 [16] 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.

3.2. 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 (30–60 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 3). Moreover, sugars were not exhausted when the concentration was higher than 50 g sugar L^{-1} (Fig. 3). Other important advantages were improved solvents concentration (Fig. 3) 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 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 4). Consequently, the butanol-to-ABE ratio was as high as 0.80.

3.3. 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 (Fig. 4). 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}.

3.4. Tolerance of C. saccharoperbutylacetonicum to butanol

The inhibitory effect of butanol on growth of C.

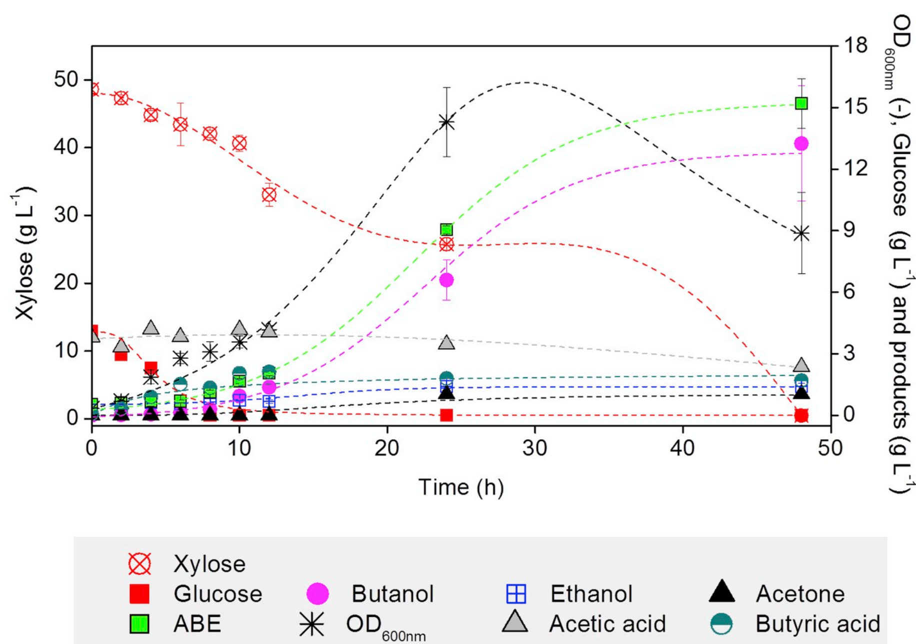

Fig. 4. Kinetics (production of ABE and acids, sugar consumption, cell growth) of *C. saccharoperbutylacetonicum* DSM 14923 cultivated in a synthetic fermentation medium containing mixed sugars (97% xylose and 7% glucose) at 50 g L^{-1}.

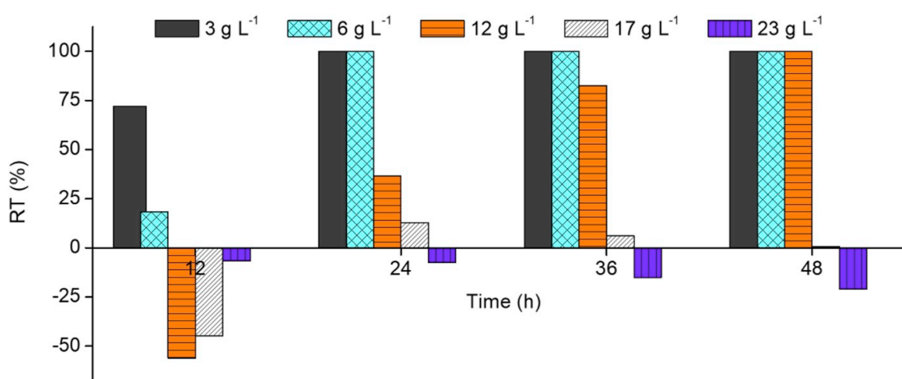

Fig. 5. Inhibitory effect of different butanol concentrations on growth of *C. saccharoperbutylacetonicum* DSM 14923 during batch fermentation of 48 h. RT is the percentage of relative tolerance as defined in Eq. (1).

Table 5
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

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) (Fig. 5). 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 of 15–17 g L^{-1}. These values are remarkably higher than the usual concentrations of 10–12 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].

3.5. 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 of 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

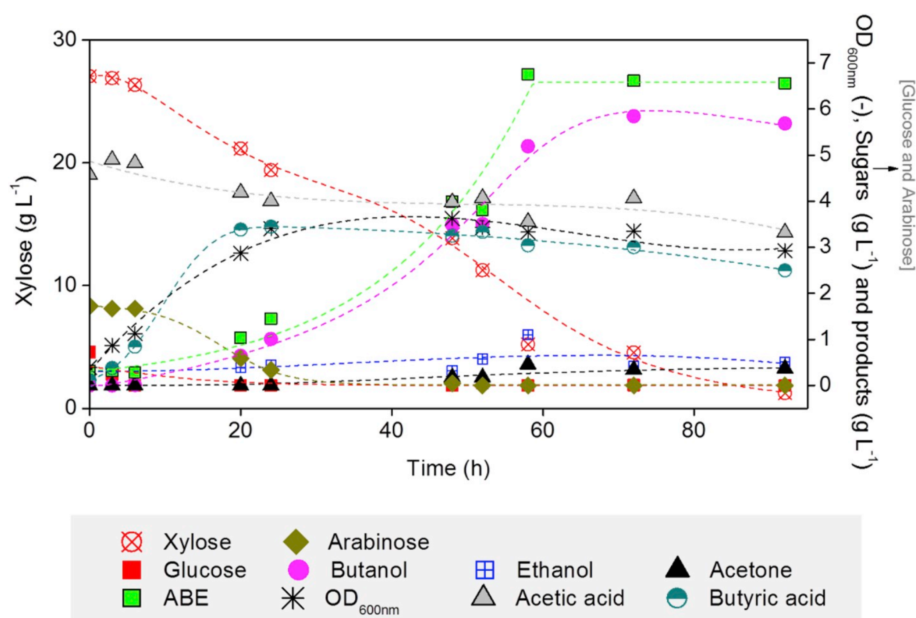

Fig. 6. Production of ABE and acids, cell growth, and sugar consumption in the ABE fermentation by *C. saccharoperbutylacetonicum* DSM 14923 using as feedstock the sugarcane bagasse hemicellulosic hydrolysate supplemented with xylose.

72 h (Table 5). 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 (Fig. 6). 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 1) 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 (Fig. 2). Moreover, acetic acid concentration decreased throughout the fermentation with hydrolysate medium (Fig. 6), 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. [23]. They also reported 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 (section 3.2). 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 technoeconomic studies with focus on this trade-off are needed.

4. 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 with 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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