ORIGINAL PAPER

Clostridium beijerinckii mutant with high inhibitor tolerance obtained by low-energy ion implantation

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Abstract *Clostridium beijerinckii* mutant strain IB4, which has a high level of inhibitor tolerance, was screened by low-energy ion implantation and used for butanol fermentation from a non-detoxified hemicellulosic hydrolysate of corn fiber treated with dilute sulfuric acid (SAHHC). Evaluation of toxicity showed *C. beijerinckii* IB4 had a higher level of tolerance than parent strain *C. beijerinckii* NCIMB 8052 for five out of six phenolic compounds tested (the exception was vanillin). Using glucose as carbon source, *C. beijerinckii* IB4 produced 9.1 g l^{-1} of butanol with an acetone/butanol/ethanol (ABE) yield of 0.41 g g^{-1}. When non-detoxified SAHHC was used as carbon source, *C. beijerinckii* NCIMB 8052 grew well but ABE production was inhibited. By contrast, *C. beijerinckii* IB4 produced 9.5 g l^{-1} of ABE with a yield of 0.34 g g^{-1}, including 2.2 g l^{-1} acetone, 6.8 g l^{-1} butanol, and 0.5 g l^{-1} ethanol. The remarkable fermentation and inhibitor tolerance of *C. beijerinckii* IB4 appears promising for ABE production from lignocellulosic materials.

Keywords Butanol · *Clostridium beijerinckii* · Inhibitor tolerance · Low-energy ion implantation

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Introduction

Butanol is a four-carbon primary alcohol used as a chemical feedstock and as a renewable fuel. In such applications, butanol is preferable to ethanol, because it is less hygroscopic, less corrosive, less volatile, and has a higher energy density [2, 8]. The biological production of acetone/butanol/ethanol (ABE) has prompted a great deal of interest in the light of diminishing oil resources worldwide and unpredictable fluctuations in petroleum prices [2, 6]. However, ABE fermentation has major disadvantages, including the high cost of the substrates, low productivity, and high recovery costs. Using renewable biomass as substrate is an attractive proposition for introducing an economically competitive biological process [14].

Lignocellulosic materials are the most abundant renewable resource on the planet, and have great potential as substrates for fermentation [9]. Currently, however, no microorganism can produce butanol efficiently from lignocellulosic biomass [16], and a range of toxic compounds, including weak acids, furan derivatives, and phenolic compounds, are generated during the hydrolysis of lignocellulosic materials [4, 10, 15]. Parekh and Wayman [12] produced ABE by SO_2-catalyzed prehydrolysis and enzyme-hydrolyzed corn stover using *Clostridium acetobutylicum* P262. Ezeji et al. [3] found that acetates, furfural, and hydroxymethylfurfural (HMF) are not inhibitory to growth and ABE production of *Clostridium beijerinckii* BA101; instead, higher solvents are produced when the medium contains a high concentration of acetate, whereas ferulic acid and syringaldehyde are potent inhibitors of ABE production by *C. beijerinckii* BA101. Qureshi et al. [16] used the *C. beijerinckii* BA101 strain to produce ABE from sulfuric acid-treated corn fiber hydrolysate (SACFH), which resulted in the production of 9.3 g l^{-1} ABE after detoxification by passage through a column of XAD-4 resin.

The low-energy ion implantation mutagenesis technique, first used in the breeding of rice seeds by Yu et al. [19], has many advantages, including a low damage rate, a higher mutation rate, and a wider spectrum of mutation compared to traditional mutation methods [5].

The objective of this study was to produce highly inhibitor-tolerant *C. beijerinckii* mutants that could produce butanol from non-detoxified hemicellulosic hydrolysate of corn fiber treated with dilute sulfuric acid (SAHHC) via low-energy ion implantation.

Materials and methods

Chemicals and gas

The phenolic compounds *p*-coumaric acid, ferulic acid, 4-hydroxybutyl acrylate (4-HBA), vanillic acid, syringaldehyde, and vanillin were purchased from Sigma Chemicals. Other chemicals were of reagent grade and were obtained from either Sinochem or Fluka Chemical. Corn steep liquor was from Anhui BBCA Biochemical Co. and N_2 was from the Nanjing Special Gases Factory (Nanjing, P.R. China).

Preparation of hemicellulosic hydrolysate of corn fiber

Corn fiber with a moisture content of 5.6% (w/v) was obtained from Shandong Zhengde Foods Ltd., China. The corn fiber was ground with a commercial plant grinder and passed through 20- to 40-mesh screens before mixing with 2% (v/v) sulfuric acid at a ratio of 1:5 (w/v). The suspension was hydrolyzed in an autoclave at 121°C for 2.5 h. The raw hydrolysate was adjusted to pH 6.0 with solid $Ca(OH)_2$ at 50°C and then filtered through filter paper to remove any solid material. After treatment at 121°C for 15 min, SAHHC contained 55.24 g l^{-1} reducing sugar and 2.77 g l^{-1} total soluble phenolic compounds (TPC) as shown in Table 1.

Active carbon was used for inhibitor removal, active carbon 2% (w/v) was added to the SAHHC and the mixture was heated at 50°C for 2 h, and then passed through filter paper to remove the active carbon.

Microorganism and culture conditions

C. beijerinckii NCIMB 8052 (purchased from the American Type Culture Collection (ATCC)) was cultivated at 35°C for 14 h in yeast extract/peptone/starch (YPS) medium (3.0 g yeast extract, 5.0 g peptone, 10.0 g soluble starch, 2.0 g ammonium acetate, 2.0 g NaCl, 3.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.1 g $FeSO_4 \cdot 7H_2O$, adjusted to pH 6.0 with 1 mol l^{-1} HCL) [7], purged with nitrogen

Table 1 The major composition of SAHHC after being sterilized at 121°C for 15 min

	Concentration (g l^{-1})
Total sugar	55.2 ± 1.6
Xylose	44.6 ± 0.9
Glucose	4.7 ± 0.4
Arabinose	3.32 ± 0.3
TPC	2.77 ± 0.48
HMF	0.38 ± 0.04
Furfural	0.66 ± 0.11

SAHHC hemicellulosic hydrolysate of corn fiber treated with dilute sulfuric acid, *TPC* total soluble phenolic compounds, *HMF* hydroxymethylfurfural

gas to remove dissolved oxygen. This culture was used for mutation studies and transferred to the butanol production medium (10% (v/v) inoculation). The agar plates used for selection of mutants had YPS medium containing 250 ml l^{-1} SAHHC, 0.02 g l^{-1} resazurin, and 0.7 g l^{-1} TPC.

Low-energy ion beam implantation

The implantation sources were produced by an ion beam implantation instrument (LZD-900 designed by the Chinese Southwestern Institute of Physics).

C. beijerinckii NCIMB 8052 cells grown in YPS medium were harvested in the exponential phase. The freshly cultured *C. beijerinckii* NCIMB 8052 cells were diluted in sterile physiologic saline solution to an optical density at 600 nm (OD_{600}) of 0.1, and 100 µl of the suspension was spread on an empty sterile Petri dish (75 mm diameter) and desiccated by a stream of filtrated air to create a dry membrane of cells. The dishes were put into the sample holder and implanted with 10 keV of energy via a beam of N^+ ions. The dose for implantation ranged from 0.4×10^{16} to 2.4×10^{16} ions cm^{-2}.

Selection of mutants

After implantation of N^+ ions, the *C. beijerinckii* NCIMB 8052 cells were washed from the plates with sterilized physiologic saline solution. The suspension was spread over six selective agar plates to isolate highly inhibitor-tolerant and high butanol-producing mutants. The highly inhibitor-tolerant and butanol-producing strains were screened for strong deoxidizing activity using resazurin (commonly used as a redox indicator). Colonies showing large haloes on the selective agar medium were selected.

Fermentation

Screw-cap bottles (100 ml) containing 60 ml of medium were used throughout these studies. In all experiments, medium was inoculated with a 10% (v/v) actively growing cell suspension, purged with nitrogen gas to remove dissolved oxygen, maintained at 35°C and incubated with no agitation or pH control for 72 h. Bottles containing 30 g l^{-1} glucose were sterilized by autoclaving at 121°C for 15 min followed by the addition of filter-sterilized P2 stock solutions: buffer solution, 50 g l^{-1} KH_2PO_4, 50 g l^{-1} K_2HPO_4, 220 g l^{-1} ammonium acetate; mineral solution, 20 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} $MnSO_4 \cdot H_2O$, 1 g l^{-1} $FeSO_4 \cdot 7H_2O$, 1 g l^{-1} NaCl; vitamin solution, 100 g l^{-1} corn steep liquor [13]. Samples of 10 ml were collected for measurement of OD_{600} and analysis of ABE after fermentation for 72 h.

Batch fermentation was done in a 3-l fermentor (Bioflo 110, USA) with an initial broth volume of 2 l containing the hemicellulosic hydrolysate of corn fiber, sterilized in an autoclave (121°C for 15 min), then filter-sterilized P2 stock solutions were added, and nitrogen gas was bubbled through the medium to remove oxygen before and after inoculation; the temperature was maintained at 35°C and there was no agitation or pH control for 90 h. During the course of fermentation, 10-ml samples were collected regularly for measurement of OD_{600} and sugars and for analysis of ABE.

Analytical methods

Dry cell weight (DCW) was computed from a curve of OD_{600} versus dry weight; an OD_{600} of 1.0 represented 260 mg dry weight. TPC was estimated by a modification of Folin–Ciocalteu's method using vanillic aldehyde and tannin as standards [17].

Glucose, xylose, and arabinose were analyzed by high-performance liquid chromatography (HPLC, Chromeleon server monitor, P680 pump, Dionex, USA). Glucose, xylose, and arabinose were measured with an RI101 refractive index detector (Shodex, USA) and an Aminex HPX-87H ion-exchange column (7.8 mm × 300 mm, BioRad, USA) with a mobile phase of 0.005 mol/l of H_2SO_4 at a flow rate of 0.6 ml/min at 55°C. The total reducing sugar concentration was measured by the 3,5-dinitrosalicyclic acid method.

Fermentation products (ABE, acetic acid, and butyric acid) were analyzed by gas chromatography (GC-2010, Shimadzu Scientific Instruments, Japan), equipped with a flame ionization detector and an InterCap WAX column (0.25 mm × 30 m, GL Sciences Inc., Japan)). Total ABE was the sum of acetone, butanol, and ethanol. The ABE yield is defined as the amount (expressed in grams per gram) of solvent produced from 1 g of total sugar.
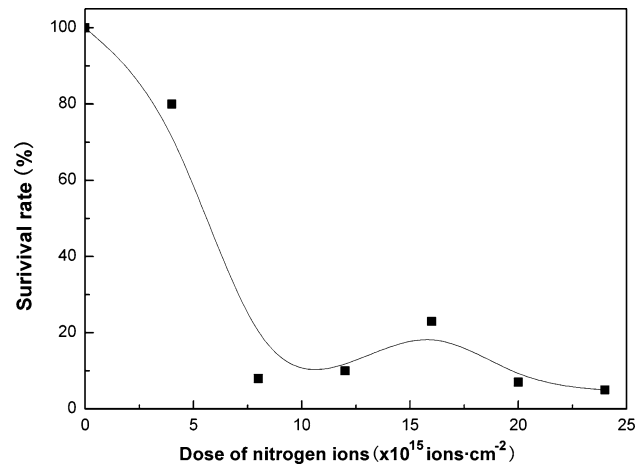
Fig. 1 The survival rate curve for *C. beijerinckii* NCIMB 8052 cells irradiated with a N^+ ion beam. The survival rate can be calculated using the number of clones scored in control plates without ion implantation as 100%

Results and discussion

Mutant dose for *C. beijerinckii* NCIMB 8052

The ion source, e.g., N^+, C^{2+}, and Ti^{2+}, can be varied depending on the specific purpose. In this study, N^+ ions were chosen as the ion source and the energy was set to 10 keV. Figure 1 shows the effect of the dose of N^+ ions beam irradiation on the survival rate of *C. beijerinckii* NCIMB 8052 cells. According to earlier reports [7, 18], the "saddle" region of the survival curve was the appropriate dose for further mutation and a dosage of 1.6×10^{16} ions cm^{-2} was chosen for this study.

Mutant screening

Strains producing high yields of butanol and ABE can be screened with resazurin for strong deoxidization activity [7]. The mutants were isolated after implantation of N^+ ions (1.6×10^{16} ions cm^{-2}) into *C. beijerinckii* NCIMB 8052 cells. Thirty colonies showing large transparent zones were selected for screw-cap bottle batch fermentation as described above. The butanol and ABE concentrations of all mutants were higher than those of the wild type (produced 7.1 g l^{-1} of butanol and 10.6 g l^{-1} of ABE). Figure 2 shows the butanol and ABE concentrations of the wild type and six mutants. *C. beijerinckii* IB4 and *C. beijerinckii* IB11 produced more than 9.0 g l^{-1} of butanol. *C. beijerinckii* IB4 was generated and inoculated into the fermentation medium to test its butanol-producing stability. During five generations of *C. beijerinckii* IB4, the average concentration of butanol was 9.1 g l^{-1} and that of total ABE was 12.4 g l^{-1}, indicating that the butanol-producing
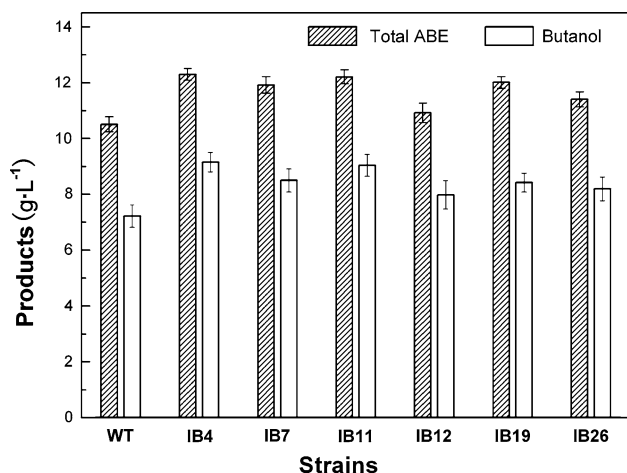
Fig. 2 Total ABE and butanol concentrations of wild type (*WT*) and mutants after N$^+$ ions implantation. Cells were grown in screw-capped bottles in fermentation medium containing 30 g l^{-1} glucose for 72 h

ability was fairly stable following mutation. In addition, the final concentration of butanol and the yield of ABE produced by *C. beijerinckii* IB4 were increased by 27 and 17% compared to the wild type.

Toxicity evaluation of model compounds

A range of toxic compounds, mainly weak acids, furan derivatives, and phenolic compounds, are generated by hydrolysis of lignocellulosic material. Ezeji et al. [3] found that acetates, furfural, and HMF were not inhibitory to ABE production in *C. beijerinckii* BA101. By contrast, phenolic compounds were potent inhibitors of ABE production in *C. beijerinckii* BA101 and were chosen as inhibitors to investigate in this study. The effects of six model phenolic compounds on cell growth and butanol production with *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* IB4 were tested by adding each of the compounds at 0.5 g l^{-1} into the fermentation medium.

As shown in Fig. 3, cell growth and butanol production were inhibited slightly by syringaldehyde, with values being similar to those of the control (free from toxic compounds). Except for syringaldehyde, the model compounds reduced cell growth by more than 76%, and a little butanol (less than 1.1 g l^{-1}) was produced by *C. beijerinckii* NCIMB 8052.

C. beijerinckii IB4 was inhibited significantly by vanillin; DCW and butanol production were decreased by 70 and 75%, respectively. Syringaldehyde slightly inhibited growth and production of butanol by *C. beijerinckii* IB4. Ferulic acid, 4-HBA, vanillic acid, and *p*-coumaric acid reduced cell growth of *C. beijerinckii* IB4 by 10, 20, 30, and 35%, respectively, and reduced its butanol production by 14, 18, 25, and 17%, respectively. Although *p*-coumaric
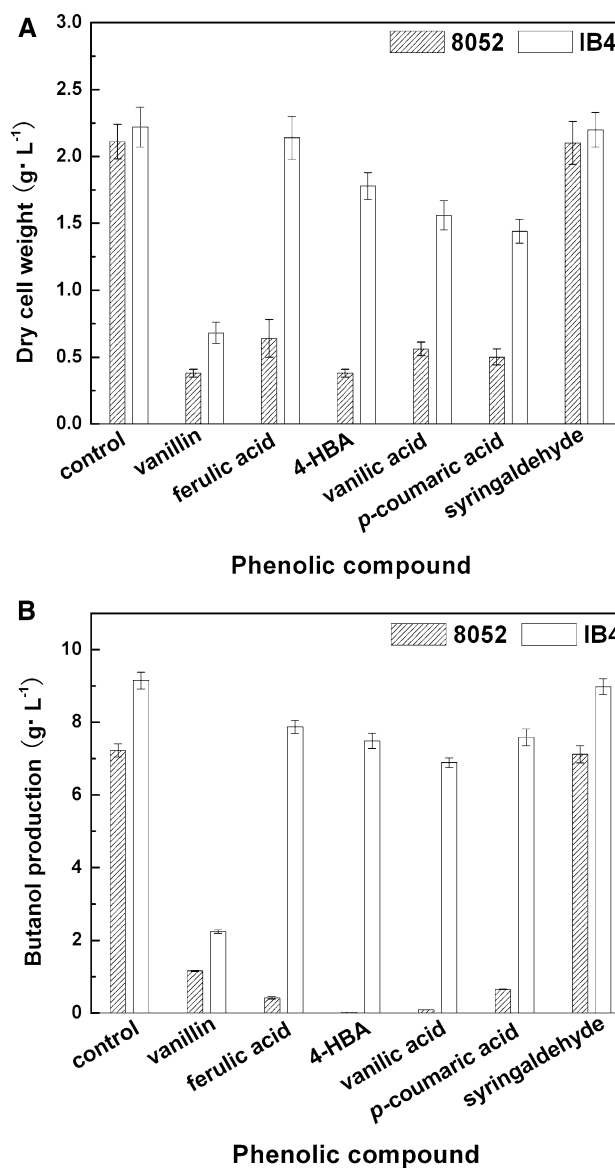
Fig. 3 Cell growth (**a**) and butanol concentration (**b**) of *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* IB4. Cells were grown in screw-capped bottles in fermentation medium containing 30 g l^{-1} glucose and each of the phenolic compounds (0.5 g l^{-1}). 8052, *C. beijerinckii* NCIMB 8052; IB4, *C. beijerinckii* IB4; 4-HBA, 4-hydroxybutyl acrylate; control, free from toxic compounds

acid was very toxic to cell growth, the level of inhibition of butanol production was quite different. In summary, the test compounds (except vanillin) did not inhibit growth or production of ABE by *C. beijerinckii* IB4 significantly.

Ezeji et al. [3] reported that syringaldehyde (1.0 g l^{-1}) had very little effect on *C. beijerinckii* BA101 growth, especially before solventogenic growth. However, syringaldehyde was a potent inhibitor of ABE production even at concentrations as low as 0.3 g l^{-1}. Conversely, Cho et al. [1] found that the level of toxicity of syringaldehyde (1.0 g l^{-1}) was very low, inhibiting cell growth and butanol production by 3 and 20%, respectively. In this study,

Table 2 Production of butanol from hydrolysates of corn fiber residue

Substrate	Hydrolysis	Inhibitor removal	Culture	ABE (g l^{-1})	Yield (g g^{-1})[b]	Productivity (g l^{-1} h^{-1})	References
Corn stover[a]	SO$_2$-catalyzed prehydrolysis and enzyme hydrolysis	None	*C. acetobutylicum* P262	25.8	0.34	1.08	Parekh and Wayman [12]
Corn fiber	Dilute sulfuric acid and enzyme hydrolysis	XAD-4 resin	*C. beijerinckii* BA101	9.3	0.39	0.1	Ezeji et al. [3]
Corn fiber	Dilute sulfuric acid and enzyme hydrolysis	None	*C. beijerinckii* BA101	1.7	–[c]	–[c]	Ezeji et al. [3]
Corn fiber	Dilute sulfuric acid	Active carbon	*C. beijerinckii* IB4	10.1	0.36	0.14	This study
Corn fiber	Dilute sulfuric acid	None	*C. beijerinckii* IB4	9.5	0.34	0.13	This study

[a] The batch fermentation was integrated with cell recycling and product recovery

[b] Yield = total ABE produced (grams)/total sugar consumed (grams)

[c] Parameters not calculated due to poor growth or fermentation

0.5 g l^{-1} syringaldehyde in the fermentation medium inhibited cell growth and butanol production by 0.5 and 2%, respectively. The results suggested that *C. beijerinckii* BA101, a mutant form of *C. beijerinckii* NCIMB 8052, had altered tolerance to syringaldehyde. The mode of inhibition of ABE production was explained by the fact that there was a twofold decrease in the expression of butanol dehydrogenase BDHII and a 7- to 11-fold increase in the stage V sporulation genes in *C. beijerinckii* during fermentation in the presence of syringaldehyde [3].

Little or no ABE was produced by *C. beijerinckii* BA101 or *C. beijerinckii* NCIMB 8052 during batch fermentation in the presence of ferulic acid [1, 3]. In this study, 2.14 g l^{-1} DCW and 7.8 g l^{-1} butanol were produced by *C. beijerinckii* IB4 in the presence of ferulic acid (0.5 g l^{-1}), similar to values obtained in the control. On the basis of the literature and the results of this study, *C. beijerinckii* IB4 is a mutant strain with high tolerance to phenolic compounds.

ABE fermentation in SAHHC-based medium

During batch fermentation, only 28–33 g l^{-1} sugars (glucose, xylose, or glucose/xylose/arabinose) was utilized by *C. beijerinckii* NCIMB 8052 (data not shown). When the non-detoxified SAHHC was used as carbon source, the fermentation medium contained 30.3 g l^{-1} reducing sugar with a total concentration of xylose, glucose, and arabinose of 28.8 g l^{-1}. Control batch fermentation was done with detoxified SAHHC (detoxified by active carbon, containing 0.3 g l^{-1} TPC) as carbon source. ABE productivity and yield of 0.14 g l^{-1} h^{-1} and 0.36 g g^{-1}, respectively, were obtained with *C. beijerinckii* IB4 at 72 h (Table 2). The concentration of total ABE was 10.1 g l^{-1} (2.3 g l^{-1} acetone, 7.2 g l^{-1} butanol, and 0.6 g l^{-1} ethanol).

As shown in Fig. 4, the concentrations of residual sugars were changed slightly after 54-h fermentation with SAHHC as carbon source; 24.1 g l^{-1} xylose, 2.6 g l^{-1} glucose, and
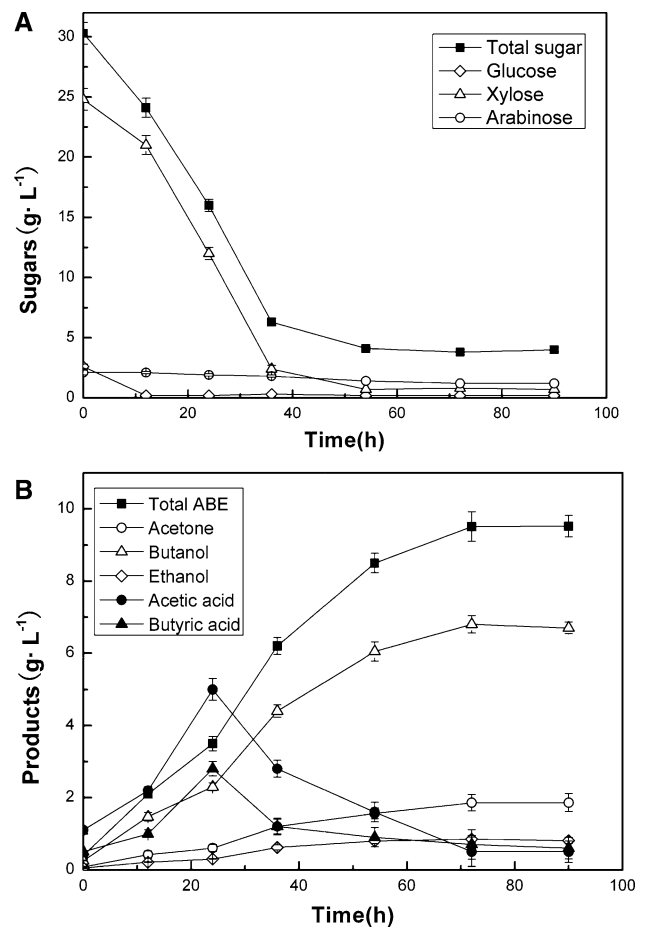
Fig. 4 Production of ABE from non-detoxified SAHHC in a batch reactor by *C. beijerinckii* IB4. Cells of *C. beijerinckii* IB4 were grown in non-detoxified SAHHC containing 30.3 g l^{-1} residual sugars for 90 h. Residual sugars in fermentation broth versus fermentation time (**a**). ABE production versus fermentation time (**b**)

0.9 g l^{-1} arabinose were consumed in 72 h (Fig. 4a). At the end of the fermentation, total ABE in the broth was 9.5 g l^{-1}, including 2.2 g l^{-1} acetone, 6.8 g l^{-1} butanol, and 0.5 g l^{-1} ethanol (Fig. 4b).

Table 3 Production of ABE from non-detoxified SAHHC in a batch reactor by *C. beijerinckii* IB4 and *C. beijerinckii* NCIMB 8052

	NCIMB 8052	IB4
TPC (g l^{-1})	1.4 ± 0.2	1.4 ± 0.2
Initial sugar (g l^{-1})	30.3 ± 0.8	30.3 ± 0.8
Residual sugar (g l^{-1})	13.1 ± 0.5	3.9 ± 0.2
Total ABE (g l^{-1})	2.4 ± 0.3	9.5 ± 0.4
Butanol (g l^{-1})	1.6 ± 0.1	6.8 ± 0.3
Acetone (g l^{-1})	0.6 ± 0.1	2.2 ± 0.3
Ethanol (g l^{-1})	0.2 ± 0.1	0.5 ± 0.1
Acetic acid (g l^{-1})	5.3 ± 0.3	0.6 ± 0.2
Butyric acid (g l^{-1})	2.8 ± 0.1	0.5 ± 0.1
Dry cell weight (g l^{-1})	2.01 ± 0.11	2.14 ± 0.12
Yield (g ABE/g total sugar)	0.14 ± 0.01	0.34 ± 0.01

Cells of *C. beijerinckii* IB4 and *C. beijerinckii* NCIMB 8052 were grown in non-detoxified SAHHC containing 30.3 g l^{-1} residual sugars for 90 h

TPC total soluble phenolic compounds

ABE productivity of 0.13 g l^{-1} h^{-1} and ABE yield of 0.34 g g^{-1} were obtained in 72 h with *C. beijerinckii* IB4; these values are similar to those of the control and the values in the literature obtained using corn fiber residues (Table 2). Qureshi [16] used the BA101 strain to produce butanol from SACFH; however, cell growth and butanol production were inhibited, and 9.3 g l^{-1} ABE and an ABE yield of 0.39 g g^{-1} were achieved only after detoxification with XAD-4 resin.

Besides corn fiber, wheat straw and wheat bran have been used for ABE production. When sulfuric acid-treated wheat bran hydrolysate (SAWBH) was used as substrate, ABE fermentation resulted in the production of 11.8 g l^{-1} ABE and a yield of 0.32 g g^{-1} in 72 h using *C. beijerinckii* ATCC 55025; however, the concentrations of the inhibitors generated from SAWBH were not reported [11].

Generally, the inhibitors and their concentrations generated during pretreatment and hydrolysis differed between lignocellulosic materials. In this study, the fermentation medium contained 1.4 g l^{-1} TPC from SAHHC. As shown in Table 3, *C. beijerinckii* NCIMB 8052 could utilize the non-detoxified SAHHC to grow well, achieving a DCW of 2.01 g l^{-1}, similar to that of *C. beijerinckii* IB4 (2.14 g l^{-1}). However, solvent production was inhibited, producing only 1.63 g l^{-1} of butanol. Acetic acid and butyric acid accumulated to concentrations of 5.3 and 2.8 g l^{-1}, respectively. In contrast, 9.5 g l^{-1} of total ABE and 6.8 g l^{-1} of butanol were produced by *C. beijerinckii* IB4, and the concentrations of acetic and butyric acids were both less than 0.7 g l^{-1}. Butanol production by *C. beijerinckii* IB4 was decreased by 9% compared to the control.

ABE-producing *Clostridia* possess an acidogenic phase and a solventogenic phase in the metabolic pathways: acetic acid and butyric acid are produced in the acidogenic phase and re-assimilated in the solventogenic phase [6]. The results showed that the inhibitors from SAHHC acted selectively on ABE production by *C. beijerinckii* NCIMB 8052. Inhibitors from SAHHC are a mixture of chemicals, some of which are toxic to cell growth, whereas others are toxic to both growth and ABE production. The underlying mechanisms of inhibition are unclear. Studies of the mechanism of high inhibitor tolerance are underway in our laboratory.

The inhibitors affect cell growth and ABE production, which makes the detoxification of inhibitors difficult and increases production costs. Improving the inhibitor tolerance of microorganisms is a promising method for facilitating industrial-scale fermentation. The results presented here demonstrate that *C. beijerinckii* IB4 is a highly inhibitor-tolerant, butanol-producing strain, which can utilize non-detoxified SAHHC to produce butanol.

Conclusions

A highly inhibitor-tolerant, butanol-producing mutant strain *C. beijerinckii* IB4 has been obtained. When non-detoxified corn fiber hemicellulosic hydrolysate was used as substrate for butanol production, 9.5 g l^{-1} ABE and 6.8 g l^{-1} butanol were produced, with an ABE yield of 0.34 g g^{-1} in 72 h. *C. beijerinckii* IB4 offers the prospect of ABE production from lignocellulosic materials.

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