***n*-Butanol production from sucrose and sugarcane juice by engineered
Clostridium tyrobutyricum overexpressing sucrose catabolism genes and *adhE2***

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Submitted to *Bioresource Technology*

Abstract

The production of *n*-butanol from sugarcane juice by metabolically engineered *Clostridium tyrobutyricum* Ct(Δ ack)-pscrBAK overexpressing *scr* operon genes (*scrB*, *scrA*, and *scrK*) for sucrose catabolism and an aldehyde/alcohol dehydrogenase gene (*adhE2*) for butanol biosynthesis was studied with corn steep liquor (CSL) as a low-cost nitrogen source. In free cell fermentation, butanol production of ~16 g/L at a yield of 0.31 ± 0.02 g/g and productivity of 0.33 ± 0.02 g/L·h was obtained from sucrose and yield of 0.24 ± 0.02 g/g and productivity of 0.30 ± 0.01 g/L·h from sugarcane juice containing sucrose, glucose and fructose. The fermentation was also studied in a fibrous bed bioreactor (FBB) operated in a repeated batch mode for 10 consecutive cycles in 10 days, achieving an average butanol yield of 0.21 ± 0.02 g/g and productivity of 0.53 ± 0.05 g/L·h from sugarcane juice, demonstrating its long-term stability without applying the antibiotic selection pressure.

Keywords: Butanol; *Clostridium tyrobutyricum*; fibrous bed bioreactor; metabolic engineering; sucrose catabolism; sugarcane juice

1. Introduction

In recent years, biological production of *n*-butanol as a renewable substitute of gasoline has attracted large attention for its higher energy density and superior fuel properties compared to ethanol (Zhao et al., 2013). However, butanol production by solventogenic clostridia in acetone-butanol-ethanol (ABE) fermentation suffered from low productivity, yield, and product titer (Zhao et al., 2013). Consequently, the high production cost of biobutanol impedes its industrial application (Green et al., 2011). Extensive efforts have been made to improve ABE fermentation by metabolic engineering of solventogenic clostridia (Cho et al., 2015; Lütke-Eversloh, 2014; Xu et al., 2015; Zheng et al., 2015) and process optimization including applying *in situ* butanol separation to reduce butanol inhibition (Lu et al., 2013; Xue et al., 2012; 2013; 2014; 2016) and using low-cost processing wastes or byproducts (Lu et al., 2012; Shao et al., 2015) and lignocellulosic biomass such as agricultural residues (Bellido et al., 2015; Jang et al., 2012). Although extensive work has been focused on using cellulosic feedstocks for biofuels production (Kumar et al., 2013a; Wang et al., 2014; Yang et al., 2015), it is difficult and expensive to hydrolyze cellulosic materials to fermentable sugars and the resulting hydrolysate containing acids and phenolic compounds derived from carbohydrate and lignin degradation are toxic to cells and severely inhibit alcohol production in ABE fermentation (Jang et al., 2012; Liu et al., 2015). Much more research and development efforts are still required for using lignocellulosic biomass hydrolysates as substrates for industrial biobutanol production (Wang et al., 2014).

On the other hand, sucrose-based substrates, such as sugarcane juice and cane molasses, are

easier to use in fermentation and cheaper than glucose and starch-based feedstocks such as corn and cassava (Filho et al., 2011; Kumar et al., 2013b; Wei et al., 2017). Although sugarcane juice and cane molasses have been used in ABE fermentation (Jiang et al., 2014; Ni et al., 2012), its economic viability is limited by the relatively low product titer, yield and productivity with conventional solventogenic clostridia (Mariano et al., 2013). Previously, we have engineered *Clostridium tyrobutyricum*, an anaerobic bacterium naturally producing butyrate as the main product, to overexpress a bifunctional aldehyde/alcohol dehydrogenase (*adhE2*) for butanol production (Yu et al., 2011). The mutant was able to produce butanol at a high titer, yield and productivity from glucose (Du et al., 2015), but not sucrose due to lacking of genes required for sucrose catabolism in its genome (Lee et al., 2016).

In the present study, three genes in the *scr* operon, *scrB*, *scrA*, and *scrK*, encoding sucrose 6-phosphate hydrolase, Enzyme II of the sucrose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), and fructokinase, respectively, and *adhE2* were cloned from *Clostridium acetobutylicum* ATCC 824 and introduced into *C. tyrobutyricum* (Δ ack), which had its acetate kinase gene (*ack*) partially knocked out (Liu et al., 2006). The mutant Ct(Δ ack)-pscrBAK was able to use sucrose, along with glucose and fructose, present in sugarcane juice to produce butanol as the main product. The fermentation kinetics with sucrose and sugarcane juice, respectively, as carbon source and corn steep liquor (CSL) as a low-cost supplemental nitrogen source (Wischral et al., 2016), was studied in free-cell fermentation. Furthermore, the mutant was also studied in a fibrous bed bioreactor (FBB), which has previously been shown to give higher butanol productivity due to higher cell density and

tolerance to butanol toxicity (Huang et al., 2004; Jiang et al., 2014), operated in a repeated batch mode with media without the antibiotic for plasmid selection, demonstrating its stability and industrial applicability for *n*-butanol production from sugarcane juice.

2. Materials and methods

2.1 Bacterial strain and culture media

The engineered strain *C. tyrobutyricum* Ct(Δ ack)-pscrBAK overexpressing *Clostridium acetobutylicum* 824 *adhE2* and *scr* operon (*scrB*, *scrA*, and *scrK*) genes was used for butanol production from sucrose in this study. To obtain Ct(Δ ack)-pscrBAK, the plasmid pscrBAK (see Figure S1 in supplemental materials) was transformed into *C. tyrobutyricum* (Δ ack) by conjugation following the previously described method (Yu et al., 2012). Positive transformants were selected on CGM agar plates containing 45 μ g /mL thiamphenicol and 250 μ g/mL cycloserine, confirmed by colony PCR screening and plasmid extraction, and maintained as a cell suspension in 15% (v/v) sterile glycerol and stored at -80 °C.

The seed culture for fermentation studies was prepared by inoculating 200 μ L of the stock culture into a serum bottle containing 50 mL Clostridial Growth Medium (CGM) supplemented with 20 g/L glucose and 30 μ g /mL thiamphenicol. The CGM medium contained (g/L): 1.0 K₂HPO₄·3H₂O, 0.5 KH₂PO₄, 2 (NH₄)₂SO₄, 0.1 MgSO₄·7H₂O, 2 yeast extract, 4 tryptone and trace minerals (Zhu and Yang, 2003). Unless otherwise noted, the medium for sucrose fermentation contained (g/L): 60 sucrose, 3 (NH₄)₂SO₄, 1.5 K₂HPO₄, 0.6 MgSO₄·7H₂O, 0.03 FeSO₄·7H₂O, 350 μ M methyl viologen (MV), and 40 g/L corn steep liquor (CSL, ~50% w/w

solids), which was obtained from a corn wet-milling plant (Ohio, USA). MV was used as an artificial electron carrier to increase intracellular NADH availability for butanol biosynthesis (Du et al., 2015). Concentrated sugarcane juice containing 44% (w/w) sucrose, 6% (w/w) glucose and 5% (w/w) fructose was obtained from a sugarcane mill (São Paulo, Brazil). Before use, it was diluted ~10-fold with water and supplemented with CSL (10 to 50 g/L) and MV (350 μ M). These media were sterilized by autoclaving at 121 °C for 30 min.

2.2 Optimization of CSL concentration in batch fermentation

The optimal concentration of CSL as the main nitrogen source was evaluated in 125-mL glass serum bottles each containing 50 mL sucrose or sugarcane juice media supplemented with various amounts of CSL (0–50 g/L). Each bottle also contained 2 g CaCO₃ (40 g/L) to buffer the fermentation pH at ~6.0. These bottles were inoculated with a seed culture of 1-day old at a volume ratio of 5 %, and incubated at 37 °C for 6 days. Liquid samples at the beginning and end of the fermentation were analyzed to determine sugars consumption and acids and alcohols production. Each condition was studied in duplicated bottles.

2.3 Free-cell fermentation in stirred-tank bioreactor

Batch fermentation kinetics of sucrose and sugarcane juice as carbon source, respectively, was studied in 1-L stirred-tank bioreactors (600 mL working volume) at 37 °C. After autoclaving, the bioreactor containing ~500 mL media was purged with filter-sterilized nitrogen gas for ~30 min to ensure anaerobiosis. The medium pH was controlled at ~6.0 by adding 40% ammonium hydroxide throughout the fermentation. Fed-batch fermentation was also studied with the initial

batch containing ~40 g/L sugar and then pulse feeding concentrated sucrose (400 g/L) or sugarcane juice into the bioreactor at ~32 h when all sugar in the medium was about to deplete. Samples were taken twice a day at regular intervals to monitor cell growth, substrates (sucrose, fructose, and glucose) consumption, and products (butanol, butyrate, acetate and ethanol) production. All batch fermentations were repeated at least once.

2.4 Immobilized-cell fermentation in FBB

To evaluate the long-term performance and strain stability, repeated-batch fermentations with cells immobilized in a fibrous bed bioreactor (FBB) (Lu et al., 2012; Jiang et al., 2014) were studied with sucrose and sugarcane juice, respectively, as carbon source and CSL as nitrogen source. The FBB (250 mL working volume), made of a glass column packed with a spirally wound cotton towel laminated with a stainless steel wire mesh as a spacer, was connected to a 1-L spinner flask, equipped with pH and temperature control, through a recirculation loop. The spinner flask containing 600 mL medium and the FBB were autoclaved separately, aseptically connected after sterilization, and then purged with nitrogen for ~30 min. To start the fermentation, a 24-h seed culture (~30 mL) prepared in CGM was inoculated into the spinner flask at a volume ratio of 5%. After the cell density in the spinner flask had reached OD₆₀₀ 6.0, cells were immobilized onto the fibrous matrix by pumping and re-circulating the fermentation broth (with cells) through the FBB. After several hours when the cell density no longer decreased, the fermentation broth was removed from the spinner flask and replaced with fresh medium to start a new batch. The repeated batch fermentation was operated for a total of 10

consecutive batches, each for 24 h. Liquid samples were taken right after each medium change, at 12 h and at 24 h right before medium change.

2.5 Analytical methods

Cells density, measured as the optical density at 600 nm (OD_{600}), in fermentation broth was analyzed with a spectrophotometer. The specific growth rate (μ) in batch fermentation was calculated based on the OD_{600} data in the exponential growth phase. Sucrose, glucose and fructose were determined by high performance liquid chromatography using a carbohydrate analysis column (Transgenomic, CARBOsep CHO-882) at 80 °C and water as the mobile phase at the flow rate of 0.5 mL/min. Acetate, butyrate, butanol, and ethanol were determined by gas chromatography (GC) as described previously (Jiang et al., 2014).

Results and discussion

3.1 Optimization of CSL concentration in batch fermentation

CSL, a byproduct of corn wet-milling, is rich in soluble proteins, amino acids, vitamins and minerals, and has been widely used as a cheap nitrogen source and nutritional supplement in industrial fermentations. Its use in clostridial fermentations improved performance by stimulating cell growth and metabolism (Choi et al., 2013; Saxena and Tanner, 2012; Wischral et al., 2016), but the supplementation level would depend on the type of substrate and microorganism used in the fermentation and must be optimized. In this study, the feasibility and optimal concentration of CSL as a low-cost nitrogen and nutrient source replacing more expensive yeast extract and tryptone were studied in serum bottles with sucrose and sugarcane

juice, respectively, as the substrate. **Figure 1** shows the effect of CSL on butanol production from sucrose after 6-day fermentation (with about 84% of sucrose consumed). As expected, increasing the CSL concentration from 10 g/L to 40 g/L in the sucrose medium also increased butanol production from 9.5 g/L to 11.7 g/L, with the corresponding butanol yield of 0.18 to 0.23 g/g and productivity of 0.07 to 0.08 g/L·h (Fig. 1A). Further increasing the CSL concentration to 50 g/L did not improve butanol production, but decreased it to 10.8 g/L at a slightly lower yield of 0.21 g/g. In general, butyrate production decreased slightly from 7.7 to 6.9 g/L with increasing the CSL concentration from 10 g/L to 50 g/L. Only small amounts of acetate and ethanol, less than 0.5 g/L, were produced in these fermentations. However, when sugarcane juice, which already contained some nitrogen sources and minerals to support good cell growth (de Souza et al., 2015; Sikder et al., 2012; Wei et al., 2017), was used as the substrate, only 10 g/L of CSL was needed to increase butanol production to 11.7 g/L (vs. 9.9 g/L for the control without CSL addition) with a butanol yield of 0.23 g/g, while further increasing CSL supplementation showed no beneficial effect (Fig. 1B). Butyrate production increased from 4.7 g/L without CSL to 5.5 g/L with 10 g/L CSL and 5.9 g/L with 50 g/L CSL. The optimal level of CSL supplementation was thus determined to be 40 g/L for sucrose and 10 g/L for sugarcane juice, respectively, as substrate in the fermentation medium.

3.2 Free-cell fermentations in stirred-tank bioreactor

Free-cell fermentations with sucrose and sugarcane juice as the substrate, respectively, supplemented with CSL as the nitrogen source were studied in stirred-tank bioreactors. **Figure 2**

shows the kinetics with the sucrose medium in batch and fed-batch fermentations. About 16.0 g/L butanol, 3.6 g/L butyrate, 1.4 g/L acetate and 1.2 g/L ethanol were produced from ~53.3 g sucrose consumed in 48 h in the batch fermentation (Fig. 2A). Similar amounts of butanol, ethanol and acetate but slightly more butyrate (5.0 g/L) were produced from sucrose in the fed-batch fermentation, which stopped at 56 h with ~21.0 g/L sucrose unused (Fig. 2B), probably because of butanol inhibition (Zhao et al., 2013). The butanol yield and productivity obtained in these fermentations were 0.31 ± 0.02 g/g and 0.33 ± 0.02 g/L·h, respectively.

Similar kinetics was obtained with sugarcane juice, which contained mainly sucrose and also small amounts of glucose and fructose. As shown in **Figure 3**, all three sugars were used simultaneously in the fermentation by Ct(Δ ack)-pscrBAK, indicating no glucose-mediated carbon catabolite repression (CCR) in the fermentation (Görke and Stülke, 2008; Yao and Shimizu, 2013). About 15.0 g/L butanol, 4.7 g/L butyrate, 1.4 g/L acetate and 1.1 g/L ethanol were produced in these fermentations, with the corresponding butanol yield and productivity of 0.24 ± 0.02 g/g and 0.30 ± 0.02 g/L·h, respectively, which were slightly lower than those obtained in the fermentations with sucrose. It is also noted that the specific growth rate for cells grown in sugarcane juice (0.071 ± 0.008 h⁻¹) was also slightly lower than that (0.077 ± 0.014 h⁻¹) in the sucrose medium. The lower specific growth rate and butanol productivity and yield could be attributed to the lower amount of CSL (10 g/L vs. 40 g/L) used in sugarcane juice fermentation and also possibly to inhibitors generated through caramelization or Maillard reaction and heavy metals (Xia et al., 2016) present in the concentrated sugarcane juice, which was obtained from fresh sugarcane juice after evaporation.

For comparison, fermentations with sucrose and sugarcane juice, respectively, as the carbon source were also conducted in CGM with yeast extract (2 g/L) and tryptone (4 g/L) as nitrogen sources, which gave much higher specific growth rates (μ) with significantly higher acids production but lower butanol production (**Table 1**). In general, yeast extract and tryptone supported faster cell growth ($\mu = 0.11\text{--}0.15\text{ h}^{-1}$ vs. $0.071\text{--}0.077\text{ h}^{-1}$ with CSL) but at the expense of lower butanol productivity ($0.21\text{--}0.28\text{ g/L}\cdot\text{h}$ vs. $0.30\text{--}0.33\text{ g/L}\cdot\text{h}$ with CSL) and yield ($0.20\text{--}0.25\text{ g/g}$ vs. $0.24\text{--}0.31\text{ g/L}\cdot\text{h}$ with CSL) due to more substrate carbon used for acids biosynthesis (butyrate: ~ 10.7 vs. $\sim 4.5\text{ g/L}$ with CSL; acetate: ~ 2.5 vs. $\sim 1.42\text{ g/L}$ with CSL). It can be concluded that CSL is a better nitrogen source than yeast extract and tryptone for butanol production from sucrose and sugarcane juice by *C. tyrobutyricum* Ct(Δ ack)-pscrBAK.

3.3 Immobilized-cell fermentation in the FBB

Repeated batch fermentations with cells immobilized in the FBB were studied to evaluate the long-term performance and stability of Ct(Δ ack)-pscrBAK, and the results are shown in **Figures 4** and **5** for fermentations with sucrose and sugarcane juice, respectively. In general, stable and consistent butanol production at $\sim 12\text{ g/L}$ and butanol yield of $0.21 \pm 0.02\text{ g/g}$ were obtained in these repeated batches, except for the first batch for reactor start up, 4th batch in the sucrose fermentation due to an operational issue, and 6th batch in the sugarcane juice fermentation. Overall, butanol productivity in these repeated batches was $0.47 \pm 0.03\text{ g/L}\cdot\text{h}$ in the sucrose fermentation and $0.53 \pm 0.05\text{ g/L}\cdot\text{h}$ in sugarcane juice, which were 42.4% – 76.7% higher than those observed in free-cell fermentations, which could be attributed to higher cell

density and tolerance to butanol cytotoxicity in the FBB (Huang et al., 2004; Jiang et al., 2014).

It should be noted that the butanol productivity was 13% higher with sugarcane juice than with sucrose in the FBB while 10% lower butanol productivity was obtained with sugarcane juice in free-cell fermentation, suggesting that cells immobilized in the FBB had better tolerance to inhibitors present in the concentrated sugarcane juice. Better cell tolerance to butanol and other inhibitors can be attributed to cell adaptation and physiological changes induced by the FBB environment, as previously reported for *n*-butanol (Huang et al., 2004; Jiang et al., 2014; Lu et al., 2012) and butyric acid fermentations (Zhu and Yang, 2003). The FBB fermentation results also confirmed that Ct(Δ ack)-pscrBAK was robust and genetically stable, even without applying antibiotic as the selection pressure for the recombinant plasmid, for butanol production from sucrose and sugarcane juice.

3.4 Comparison to other studies

Ct(Δ ack)-pscrBAK overexpressing *adhE2* and three *scr* operon genes (*scrB*, *scrA*, and *scrK*) was able to produce butanol from sucrose present in sugarcane juice at a high productivity of 0.30 g/L·h in free-cell fermentation and 0.53 g/L·h in the FBB, which were 15% – 56% higher than those in similar fermentations with *C. acetobutylicum* JB200 (Jiang et al., 2014), probably because the latter was subject to glucose repression. Glucose and fructose present in sugarcane juice and molasses are the preferred substrates over sucrose and can cause CCR in common solventogenic clostridia including *C. acetobutylicum* and *C. beijerinckii* (Reid et al., 1999; Tangney and Mitchell, 2000). It should be noted that there were few studies on the analysis of

sucrose metabolism in clostridia (Tangney and Mitchell, 2000; Reid et al, 1999) and *E. coli* (Sabri et al., 2013). To date, most of the glucose CCR studies have focused on its effects on xylose utilization and strategies to overcome the CCR in fermentation with glucose and xylose as co-substrates (Gawand et al., 2013; Matsuoka and Shimizu, 2013; Wei et al., 2016; Yu et al., 2015b). The work reported in this study was the first demonstrating the use of heterologous *scr* genes to enable sucrose utilization in a bacterium (*C. tyrobutyricum*) without being subject to CCR.

Ct(Δ ack)-pscrBAK also compared favorably to other solventogenic strains, including *C. acetobutylicum* ATCC 824 (Kittithanesuan and Phisalaphong, 2015), *C. beijerinckii* NCP P260 (Shaheen et al., 2000) and *C. saccharobutylicum* DSM 13864 (Ni et al., 2012), which produced butanol from sugarcane juice or cane molasses at a lower titer of 12 – 15 g/L due to lower butanol tolerance (see **Table 2**). Furthermore, Ct(Δ ack)-pscrBAK produced *n*-butanol as the main product and only a small amount of ethanol and no acetone, which would be much easier for butanol to be separated and purified in downstream processing, offering another advantage over conventional ABE fermentation (Du et al., 2015).

It should be noted that the performance of sucrose fermentation by Ct(Δ ack)-pscrBAK also compared favorably to other engineered strains of *C. tyrobutyricum* in using different low-cost substrates, including Ct(Δ ack)-pGluI with maltose and soluble starch (Yu et al., 2015a) and Ct(Δ ack)-pTBA with glucose and xylose present in soybean hull hydrolysate (Yu et al., 2015b) (see Table 2). Since sucrose and sucrose-based feedstock are cheaper than glucose and starch-based substrates (Jiang et al., 2014) and cellulosic feedstocks are difficult and expensive

to be hydrolyzed to fermentable sugars (Jang et al., 2012; Wang et al., 2014), using Ct(Δ ack)-pscrBAK to ferment sugarcane juice supplemented with CSL should provide an economically viable and competitive process for industrial biobutanol production.

4. Conclusions

C. tyrobutyricum Ct(Δ ack)-pscrBAK was engineered to express *scrB*, *scrA*, and *scrK* for sucrose catabolism and *adhE2* for butanol biosynthesis. The engineered strain was able to use sucrose simultaneously with glucose and fructose present in sugarcane juice without subjecting to CCR. The fermentation of sugarcane juice supplemented with corn steep liquor (CSL) by Ct(Δ ack)-pscrBAK gave a high butanol titer, yield and productivity, providing an economically viable process for biobutanol production.

Acknowledgements

Financial support from the National Science Foundation STTR program (IIP-1026648) and China Scholarship Council for research visit of Zhang (201406350172) at OSU are acknowledged.

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Table 1. Kinetics of free-cell fermentation of Ct(Δ *ack*)-pscrBAK with sucrose or sugarcane juice as carbon source and CSL or YE and Tryp as nitrogen source.

Carbon / Nitrogen source	μ (h ⁻¹)	Butanol (g/L)	Butanol Yield (g/g)	Productivity (g/L·h)	Butyrate (g/L)	Acetate (g/L)	Ethanol (g/L)
Sucrose							
CSL 40	0.077±0.014	16.0±0.2	0.31±0.02	0.33±0.02	4.3±0.7	1.5±0.1	1.1±0.2
YE 2, Tryp 4	0.15±0.02	16.1±1.1	0.25±0.02	0.28±0.02	10.2±1.5	2.5±0.3	1.3±0.0
Sugarcane juice							
CSL 10	0.071±0.008	15.0±0.7	0.24±0.02	0.30±0.02	4.7±0.2	1.4±0.2	1.1±0.1
YE 2, Tryp 4	0.11±0.02	11.9±0.4	0.20±0.03	0.21±0.01	11.2±1.2	2.6±0.3	1.1±0.2

CSL: corn steep liquor, g/L; YE: yeast extract, g/L; Tryp: tryptone, g/L.

Table 2. Comparison of Ct(Δ ack)-pscrBAK with other solventogenic clostridia strains for butanol production

Strain / Substrate (N source)	Condition	Titer (g/L)	Yield (g/g)	Productivity (g/L h)	References
<i>C. beijerinckii</i> NCP P260					Shaheen et al., 2000
Cane molasses	Free cell	14.2	0.21	NA	
<i>C. saccharobutylicum</i> DSM 13864					Ni et al., 2012
Cane molasses	Free cell	11.9	0.22	0.33	
<i>C. acetobutylicum</i> ATCC 824					Kittithanesuan and
Sugarcane juice (thin-shell silk cocoons)	Free cell	14.0	0.19	0.11	Phisalaphong, 2015
	IMC	12.9	0.20	0.28	
<i>C. acetobutylicum</i> JB200					Jiang et al., 2014
Sucrose (YE, Tryp)	Free cell	14.9	0.21±0.02	0.25±0.03	
	FBB	16.8	0.21±0.04	0.32±0.03	
Sugarcane juice (YE, Tryp)	Free cell	18.5	0.20±0.02	0.26±0.05	
	FBB	16.6	0.20±0.01	0.34±0.01	
<i>C. tyrobutyricum</i> Δ ack-adhE2-pscrBAK					This study
Sucrose (CSL, 4%)	Free cell	16.0±0.2	0.31±0.02	0.33±0.02	
	FBB	11.2±0.8	0.22±0.02	0.47±0.03	
Sugarcane juice (CSL, 1%)	Free cell	15.7±0.2	0.24±0.02	0.30±0.01	
	FBB	12.8±1.2	0.21±0.02	0.53±0.05	
<i>C. tyrobutyricum</i> Δ ack-adhE2					Du et al., 2015
Glucose (YE, Tryp)	Free cell	14.6	0.25	0.26	
<i>C. tyrobutyricum</i> Δ ack-adhE2-agluI					Yu et al., 2015a
Maltose (YE, Tryp)	Free cell	17.2±0.2	0.20±0.02	0.29±0.02	
Soluble starch (YE, Tryp)	Free cell	16.3±0.2	0.17±0.02	0.19±0.03	
<i>C. tyrobutyricum</i> Δ ack-adhE2-pTBA					Yu et al., 2015b
SHH (YE, Tryp)	Free cell	15.7±0.4	0.24±0.02	0.29±0.01	

FBB: fibrous bed bioreactor; IMC: immobilized cell; NA: not available; SHH: soybean hull hydrolysate containing 45 g/L glucose and 45 g/L xylose

List of Figures

Figure 1. Effects of CSL on fermentation kinetics of *C. tyrobutyricum* Ct(Δ ack)-pscrBAK grown on sucrose (A) and sugarcane juice (B) in serum bottles with 40 g/L CaCO₃, 350 μ M MV, and no thiamphenicol. Acetate and ethanol were less than 0.5 g/L (data not shown). Average values with standard deviations indicated by error bars from two independent bottles are shown.

Figure 2. Kinetics of free-cell fermentations of sucrose by *C. tyrobutyricum* Ct(Δ ack)-pscrBAK in stirred-tank bioreactors at pH 6.0 and 37 °C. The medium also contained 40 g/L CSL and 350 μ M MV, but no thiamphenicol. A. Batch fermentation; B. Fed-batch fermentation.

Figure 3. Kinetics of free-cell fermentations of sugarcane juice by *C. tyrobutyricum* Ct(Δ ack)-pscrBAK in stirred-tank bioreactors at pH 6.0 and 37 °C. The medium also contained 10 g/L CSL and 350 μ M MV, but no thiamphenicol. A. Batch fermentation; B. Fed-batch fermentation.

Figure 4. Repeated batch fermentations of sucrose by *C. tyrobutyricum* Ct(Δ ack)-pscrBAK immobilized in a FBB. The media also contained 40 g/L CSL and 350 μ M MV but no thiamphenicol. A. Time course profiles of sucrose and products in the fermentation medium; B. butanol yield and productivity.

Figure 5 Repeated batch fermentations of sugarcane juice by *C. tyrobutyricum* Ct(Δ ack)-pscrBAK immobilized in a FBB. The media also contained 10 g/L CSL and 350 μ M MV but no thiamphenicol. A. Time course profiles of sugars and products in the fermentation medium; B. butanol yield and productivity.

A

B

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Graphical Abstract