Metabolic engineering of *Clostridium tyrobutyricum* for enhanced butyric acid production from glucose and xylose

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Abstract

Clostridium tyrobutyricum is a promising microorganism for butyric acid production. However, its ability to utilize xylose, the second most abundant sugar found in lignocellulosic biomass, is severely impaired by glucose-mediated carbon catabolite repression (CCR). In this study, CCR in *C. tyrobutyricum* was eliminated by overexpressing three heterologous xylose catabolism genes (*xylT*, *xylA* and *xlyB*) cloned from *C. acetobutylicum*. Compared to the parental strain, the engineered strain Ct-pTBA produced more butyric acid (37.8 g/L vs. 19.4 g/L) from glucose and xylose simultaneously, at a higher xylose utilization rate (1.28 g/L·h vs. 0.16 g/L·h) and efficiency (94.3% vs. 13.8%), resulting in a higher butyrate productivity (0.53 g/L·h vs. 0.26 g/L·h) and yield (0.32 g/g vs. 0.28 g/g). When the initial total sugar concentration was ~120 g/L, both glucose and xylose utilization rates increased with increasing their respective concentration or ratio in the co-substrates but the total sugar utilization rate remained almost unchanged in the fermentation at pH 6.0. Decreasing the pH to 5.0 significantly decreased sugar utilization rates and butyrate productivity, but the effect was more pronounced for xylose than glucose. The addition of benzyl viologen (BV) as an artificial electron carrier facilitated the re-assimilation of acetate and increased butyrate production to a final titer of 46.4 g/L, yield of 0.43 g/g sugar consumed, productivity of 0.87 g/L·h, and acid purity of 98.3% in free-cell batch fermentation, which were the highest ever reported for butyric acid fermentation. The engineered strain with BV addition thus can provide an economical process for butyric acid production from lignocellulosic biomass.

Keywords: Butyric acid; Carbon catabolite repression; *Clostridium tyrobutyricum*; Metabolic engineering; Xylose

1 Introduction

2 *Clostridium tyrobutyricum*, a Gram-positive, chemotrophic, spore-forming anaerobe, can
3 produce butyric acid as the main product at a high titer, yield, and productivity from glucose and
4 xylose (Jiang et al., 2010; 2011), two main sugars present in lignocellulosic biomass hydrolysates.
5 *C. tyrobutyricum* thus has a great potential for industrial production of bio-based butyric acid,
6 which has wide applications in food, animal feed, chemical and pharmaceutical industries (Dwidar
7 et al., 2012; Yang et al., 2013; Zhang et al., 2009) with a global market of >80,000 metric tons per
8 year (Wang et al., 2016). However, like other clostridia, xylose utilization by *C. tyrobutyricum* is
9 inhibited or repressed by glucose, a phenomenon called carbon catabolite repression (CCR) widely
10 observed in bacteria and yeasts (Görke and Stülke, 2008; Yao and Shimizu, 2013; Yu et al., 2015).
11 Consequently, butyric acid production from lignocellulosic biomass is hindered by the inefficient
12 utilization of xylose in the hydrolysates (Baroi et al., 2015a; 2015b; Liu et al., 2013; Wei et al.,
13 2013; Zhu et al., 2002), which limits its commercial application.

14 Xylose catabolism in *Clostridium* usually involves a D-xylose-proton symporter (XylT),
15 xylose isomerase (XylA), and xylulokinase (XylB) (see **Figure 1**), which are regulated at the
16 transcriptional level by a putative xylose transcriptional repressor XylR and the catabolite control
17 protein A (CcpA) (Gu et al., 2014). XylR could repress *xylT*, *xylA*, and *xylB* genes (Rodionov et al.,
18 2001), which are present in the same operon in the genome of *C. tyrobutyricum* ATCC 25755
19 (Jiang et al., 2013), whereas CcpA is the global transcriptional regulator in CCR (Lorca et al., 2005;
20 Moreno et al., 2004). The enzyme II (GlcG) in the glucose phosphoenolpyruvate (PEP)-dependent
21 phosphotransferase system (PTS) also plays an important role in the induction of CCR (Tangney

and Mitchell, 2007; Xiao et al., 2011). Previous studies showed that butanol production and xylose utilization in the presence of glucose were greatly enhanced in solventogenic *C. acetobutylicum* (Xiao et al., 2011) and *C. tyrobutyricum* (Yu et al., 2015) overexpressing *xylT*, *xylA* and *xylB*, which appeared to be an effective metabolic engineering strategy to eliminate or alleviate the glucose-mediated CCR.

The objective of the present study was to engineer *C. tyrobutyricum* for butyric acid production from both glucose and xylose. Three genes, *xylT* (CAC1345), *xylB* (CAC2612), and *xylA* (CAC2610) from *C. acetobutylicum* were cloned and overexpressed in *C. tyrobutyricum* under the control of a thiolase promoter. We hypothesized that constitutively expressing heterologous XylT, XylB, and XylA could circumvent the CCR regulated by native XylR and CcpA, thus allowing efficient co-utilization of xylose and glucose, which was confirmed in batch fermentations carried out in serum bottles and bioreactors in this study. The mutant Ct-pTBA overexpressing these three genes was also characterized for its ability to use xylose at various pH values and glucose/xylose ratios in batch fermentation. Furthermore, we also studied the effects of benzyl viologen (BV) as an artificial electron carrier on increasing NADH availability and butyric acid production from glucose and xylose, and demonstrated that adding a small amount of BV could increase butyric acid production by re-assimilating acetate, achieving a high butyrate yield and purity needed for economic production of bio-based butyric acid. These results indicated that *C. tyrobutyricum* Ct-pTBA is a promising strain for butyric acid production from abundant, renewable lignocellulosic biomass.

2. Materials and methods

2.1. Cultures and media

The bacterial strains, plasmids and primers used in this study are listed in **Table 1**. *C. tyrobutyricum* strains were cultivated anaerobically at 37 °C in Clostridial Growth Medium (CGM) described previously (Liu et al., 2006; Zhu et al., 2005). *E. coli* DH5 α and *E. coli* CA434 were grown aerobically at 37 °C with agitation at 250 rpm in Luria-Bertani (LB) media or on LB agar plates. All media were autoclaved (121 °C for 30 min) and then supplemented with corresponding antibiotics (30 μ g/mL chloramphenicol, 50 μ g/mL kanamycin, 30 μ g/mL thiamphenicol or 250 μ g/mL cycloserine), unless otherwise noted.

2.2. Plasmid construction

Three genes, *xylT* (CAC1345), *xylB* (CAC2612), and *xylA* (CAC2610), in the xylose catabolic pathway from *C. acetobutylicum* ATCC 824 were used in constructing the recombinant plasmid pTBA (see **Figure S1** in Supplementary Materials). First, *xylTB* and *xylA* were separately PCR-amplified from Ct(Δ *ack*)-pTBA (Yu et al., 2015) using primers shown in **Table 1**. To construct the recombinant plasmid pTBA, pMAD22 (Yu et al., 2011) was first digested with *Bam*HI and *Sac*II to remove *adhE2*, and then ligated sequentially with *xylTB* and *xylA* using the Clontech infusion cloning kit (Clontech Laboratories, Inc., Mountain View, CA). All recombinant plasmids were first transformed into *E. coli* DH5 α for amplification. Plasmids were then extracted using Qiagen QIAprep Mini-Prep plasmid purification kit (Valencia, CA) and confirmed by checking plasmid size and digestion pattern.

2.3. Transformation

The transformation of pTBA into *C. tyrobutyricum* was done by conjugation carried out in an anaerobic chamber following previously described procedures (Yu et al., 2012). First, pTBA was transformed into *E. coli* CA434 cells, which were cultivated in LB medium containing 30 µg/mL chloramphenicol and 50 µg/mL kanamycin at 37 °C overnight. The *E. coli* CA434 cells were collected by centrifugation, washed with sterile phosphate-buffered saline (PBS), mixed with *C. tyrobutyricum* Ct cells, and the mixture was then pipetted onto CGM agar plates. After 12 h, cells were collected with CGM and re-plated on selective CGM plates containing 250 µg/mL cycloserine and 30 µg/mL thiamphenicol. The plates were incubated at 37 °C until visible colonies were formed. Positive transformants were confirmed by colony PCR and plasmid extraction followed with gel electrophoresis to verify the plasmid size. The selected transformant was maintained in CGM containing 30 µg/mL thiamphenicol and 15% glycerol, and stored at -80 °C.

2.4. Fermentation kinetics studies

Batch fermentation kinetics with glucose, xylose, and glucose/xylose mixture, respectively, as carbon sources was first studied in serum bottles (50 ml CGM buffered with 40 g/L CaCO₃) and then in 1-L bioreactors (600 ml CGM) with pH controlled with ammonia hydroxide (30%, v/v) at 37 °C. The serum bottles and bioreactors were sterilized by autoclaving at 121 °C for 30 min, sparged with nitrogen for ~30 min to reach anaerobiosis, and then inoculated with an active seed culture (~24 h) at a volume ratio of 5%. For Ct-pTBA, 30 µg/mL thiamphenicol was also added into the medium at the time of inoculation. Batch fermentation kinetics was studied with glucose

and xylose at different ratios and at various pH values (6.0, 5.5, and 5.0). The effects of BV and acetic acid addition on butyric acid production were also studied. Liquid samples were taken at regular time intervals to monitor cell growth, sugar consumption and acid production.

2.5. Analytical methods

Cell density was monitored by measuring the optical density (OD) at 600 nm using a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). Glucose, xylose, acetic acid, lactic acid and butyric acid were analyzed by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Columbia, MD) with an organic acid analysis column (HPX-87H, Bio-Rad, Hercules, CA) at 65 °C and 0.005 M sulfuric acid as the mobile phase at 0.6 mL/min. Details can be found elsewhere ([Liu and Yang, 2006](#)).

3. Results

3.1. Batch fermentation of glucose and xylose by Ct and Ct-pTBA

Batch fermentation of glucose, xylose and glucose/xylose (1:1), respectively, as carbon sources were first evaluated in serum bottles with 40 g/L calcium carbonate to buffer the culture pH at ~5.5. The results with *C. tyrobutyricum* strains Ct and Ct-pTBA are summarized in **Table 2**. In general, both strains could use xylose as the sole carbon source, although xylose fermentation was much slower compared to glucose (see **Figure S2** in Supplementary Materials), which could be attributed to the fact that less acetate and ATP were produced from xylose ([Zhu and Yang, 2004](#)). Compared to Ct, Ct-pTBA was significantly faster in utilizing xylose (xylose uptake rate: 0.32 ± 0.03 vs. 0.24 ± 0.03 g/L·h), but slower in utilizing glucose (glucose uptake rate: 0.46 ± 0.02 vs.

0.64 $\pm$ 0.02 g/L·h). The slower growth on glucose by Ct-pTBA could be attributed to the added metabolic burden to overexpress XylT, XylA, and XylB. However, when glucose and xylose were both present in the medium, xylose utilization was inhibited in the fermentation with Ct, while simultaneous utilization of xylose and glucose was observed in the fermentation with Ct-pTBA. Apparently, xylose utilization was inhibited in Ct due to glucose-induced CCR, which was alleviated in Ct-pTBA overexpressing *xylT*, *xylA*, and *xylB*. It should be noted that xylose was not utilized by Ct even after all glucose had been consumed in the fermentation. This was probably due to that xylose fermentation was sensitive to the pH (Zhu and Yang, 2004), which decreased from the initial 6.5 to ~4.8 in serum bottles and thus stopped the fermentation due to pH limitation.

The effect of overexpressing *xylT*, *xylA*, and *xylB* on the fermentation with glucose and xylose as co-substrates was further studied in bioreactor with pH controlled at 6.0. As shown in **Figure 2A**, Ct consumed all glucose in 38 h but only 13.8% of the xylose in 75 h, producing 19.4 g/L butyric acid at a low overall productivity of 0.26 g/L·h and yield of 0.28 g/g. In contrast, Ct-pTBA consumed all glucose and 94.3% xylose simultaneously and produced 37.8 g/L butyric acid in 72 h (**Figure 2B**), at an overall productivity of 0.53 g/L·h and yield of 0.32 g/g, which were 104% and 14%, respectively, higher than those by Ct. Excluding the periods of the initial lag and late stationary phases, Ct had a higher butyrate productivity of 0.92 g/L·h from glucose compared to 0.76 g/L·h by Ct-pTBA from the mixture of glucose and xylose. Compared to Ct, glucose consumption was slowed down (rate: 1.16 g/L·h vs. 1.60 g/L·h) while xylose consumption was accelerated (rate: 1.28 g/L·h vs. 0.16 g/L·h) in Ct-pTBA. Although cell growth seemed to be slower at the beginning for Ct-pTBA, both Ct and Ct-pTBA had a similar specific growth rate of

1 $\sim 0.14 \pm 0.01 \text{ h}^{-1}$ in the fermentation (see **Figure S3A** in Supplementary Materials), suggesting that
2 the addition of antibiotic and overexpressing XylT, XylA, and XylB did not negatively impact cell
3 growth in the fermentation by Ct-pTBA.

4 **3.2. Effects of pH**

5 Xylose fermentation by *C. tyrobutyricum* is affected by the pH (Zhu and Yang, 2004). The
6 effects of pH on glucose and xylose utilization by Ct-pTBA were thus studied in bioreactor with its
7 pH controlled at 5.0, 5.5 and 6.0, respectively. About equal amount ($\sim 60 \text{ g/L}$) of glucose and
8 xylose was used as co-substrates in the fermentation. The fermentation profiles are shown in
9 **Figure 3** and corresponding kinetic parameters are summarized in **Table 3**. At pH 6.0, the strain
10 consumed glucose and xylose simultaneously, and all glucose and 95% of xylose were consumed
11 in 74 h, producing 37.8 g/L butyric acid at a productivity of $\sim 0.86 \text{ g/L}\cdot\text{h}$ and yield of 0.32 g/g. At
12 pH 5.5, all glucose and 66% of xylose were consumed in 84 h, producing 30.2 g/L butyric acid at a
13 productivity of $\sim 0.55 \text{ g/L}\cdot\text{h}$ and yield of 0.29 g/g. When the pH decreased to 5.0, the fermentation
14 was significantly slower with only 58% of glucose and 29% of xylose consumed and 15.6 g/L
15 butyric acid produced in 84 h. The much lower rates in consuming glucose ($0.66 \text{ g/L}\cdot\text{h}$) and
16 xylose ($0.38 \text{ g/L}\cdot\text{h}$) and in producing butyrate ($0.34 \text{ g/L}\cdot\text{h}$) at pH 5.0 could be attributed to the
17 cytotoxicity of the undissociated butyric acid ($pK_a = 4.82$) (Wu and Yang, 2003), which inhibited
18 cell growth and metabolism by disrupting the transmembrane pH gradient and reducing ATP level
19 and activities of acid-forming enzymes (Zhu and Yang, 2003; Zigova and Sturdik, 2000). Our
20 result was in good agreement with the observation of a decreased xylose consumption rate from

~1.1 g/L·h at pH 6.3 to ~0.56 g/L·h at pH 5.3 in batch fermentation by *C. tyrobutyricum* (Zhu and Yang, 2004). It should be noted that the inhibition seemed to be more pronounced on xylose than on glucose utilization rate. The former reduced 73% (from 1.40 g/L·h at pH 6.0 to 0.38 g/L·h at pH 5.0) while the latter reduced 45% (from 1.20 g/L·h at pH 6.0 to 0.66 g/L·h at pH 5.0). The higher pH sensitivity for xylose utilization could be attributed to the fact that xylose transport is mainly through the xylose-proton symporter while glucose is via PEP-dependent PTS. The results suggest that pH control at ~6.0 is crucial for efficient co-utilization of glucose and xylose by *C. tyrobutyricum* Ct-pTBA.

3.3. Effects of glucose/xylose ratio

The fermentation kinetics of Ct-pTBA with glucose, xylose and mixtures of glucose and xylose at different ratios (3:1, 1:1, and 1:3), representing the sugar compositions found in different lignocellulose hydrolysates, were investigated in bioreactor at pH 6.0 (see **Figure 4**), and the results are summarized in **Table 3**. As expected, glucose fermentation (**Fig. 4A**) was faster than xylose fermentation (**Fig. 4B**) because glucose metabolism could provide more ATP for cell growth. Consequently, xylose fermentation gave a significantly lower specific growth rate (0.09 h⁻¹ vs. 0.16 h⁻¹), sugar utilization rate (1.78 g/L·h vs. 2.79 g/L·h), and butyrate productivity (0.51 g/L·h vs. 0.86 g/L·h) compared to glucose fermentation (see **Table 3**). However, the final butyric acid titer (~36.5 g/L) and yield (~0.30 g/g) were comparable for both xylose and glucose as the sole carbon source in the fermentation.

1 In the fermentations with glucose and xylose as co-substrates, both the specific growth rate and
2 butyrate productivity generally increased with increasing the glucose/xylose ratio (**Fig. 4E**).
3 Interestingly, both glucose and xylose consumption rates increased with increasing their respective
4 ratio or concentration in the substrate mixture, but the total sugar consumption rate was almost the
5 same at ~2.5 g/L·h for all three glucose/xylose ratios studied. These results suggested that the
6 catabolism of glucose and xylose in Ct-pTBA was limited by the sugar uptake (or influx) rate,
7 which apparently increased with the extracellular sugar concentration. An earlier study also
8 reported that xylose utilization by *C. thermohydrosulfuricum*, which was not subjected to
9 glucose-mediated CCR, increased with xylose concentration (0–50 mM) and suggested that xylose
10 uptake was a facilitated diffusion process (Cook et al., 1993). The increased xylose utilization
11 rate could also be attributed to increased activities of xylose isomerase and xylulose kinase, which
12 in *Clostridium* sp. strain BOH3 were found to increase proportionally with the extracellular xylose
13 concentration (Xin et al., 2014). The fact that both glucose and xylose utilization rates increased
14 with increasing their respective concentration also suggested that xylose transport was through
15 both XylT and non-XylT while glucose transport involved both PTS and non-PTS (see Fig. 1),
16 which remains to be verified experimentally, however.

17 **3.4. Effect of benzyl viologen (BV) on butyric acid production**

18 Butyric acid biosynthesis in *C. tyrobutyricum* requires NADH and is accompanied with acetate
19 biosynthesis, which not only provides ATP needed for cell growth but also balances the redox.
20 Previous studies have shown that the addition of an artificial electron carrier such as methyl

viologen (MV) in the fermentation medium can provide more NADH, via reduced hydrogen production, for the biosynthesis of more reduced metabolites such as butyrate (vs. acetate) and butanol (Choi et al., 2012; Du et al., 2014; Rao and Mutharasan, 1987). Therefore, an artificial electron carrier, BV, was studied for its effects on butyric acid production and sugar utilization in the co-fermentation of glucose and xylose by Ct-pTBA. Because of its inhibition effect on cell growth, BV was added at the late exponential or early stationary phase in the fermentation. As shown in **Figure 5**, the addition of BV did not seem to affect sugar utilization but induced acetate re-assimilation and increased butyrate production. Compared to the fermentation without BV addition (see Fig. 2B), a significantly higher final butyric acid titer of 39.8 g/L (vs. 37.8 g/L) and yield of 0.36 g/g (vs. 0.32 g/g) were obtained in the fermentation with 0.75 μ M BV (**Fig. 5A**). In fact, the butyrate yield from sugars was 0.42 g/g after BV addition. The increased butyrate production could be attributed to the re-assimilation of acetic acid, which decreased from 2.74 g/L at 38 h (the time BV was added) to zero at 70 h. Interestingly, upon the depletion of acetate, lactic acid production started and reached 0.45 g/L at the end of the fermentation. The production of lactic acid, which was observed only in the presence of BV and in the absence of acetate, was probably for cells to consume NADH for redox balance.

Further experiments with adding both acetate (18 g/L) and BV (3.75 μ M) to the fermentation medium verified that acetate re-assimilation occurred without lactate production while butyric acid production increased to 46.4 g/L (**Fig. 5B**). Butyric acid yield from sugars increased from 0.30 g/g before BV addition to 0.52 g/g from both sugars and acetate consumed in the presence of 3.75 μ M BV. The theoretical yield of butyric acid from glucose in the fermentation is 0.8

mol/mol (0.39 g/g) when acetate (0.4 mol/mol) and carbon dioxide (2 mol/mol) are also produced as co-products (Wang et al., 2014; Zhang et al., 2009). In contrast, butyrate formation from acetate has a theoretical yield of 0.73 g/g (0.5 mol/mol), which is 87.2% higher than that from glucose, because no substrate carbon is lost in carbon dioxide. In addition to the higher butyrate yield, butyrate was produced as the only main product in the fermentation with a high acid purity of 98.3%, which was not only much higher than ~80% in typical butyric acid fermentation but also higher than those obtained with metabolically engineered butyrate-producing *C. acetobutylicum* (88%) (Jang et al., 2014) and *E. coli* (97.6%) (Lim et al., 2013). It should be noted that only 77%–84% of the xylose was consumed while all glucose was utilized in these fermentations. This was because butyric acid inhibition was more pronounced in xylose fermentation than in glucose fermentation (Jiang et al., 2010; Liu and Yang, 2006).

Figure 6 illustrates the metabolic pathways involved in lactate, acetate and butyrate biosynthesis, and possible effects of BV on metabolic flux distributions among various metabolites and NADH balance. **Table 4** shows the carbon balance in the co-fermentation of glucose and xylose (1:1) by Ct and Ct-pTBA. Ct used glucose as the preferred substrate, accounting for 87.8% (vs. 12.2% for xylose) of the carbon consumed in the fermentation. In contrast, Ct-pTBA used both glucose and xylose almost equally at 50% each. When BV was present, more glucose (55%) was consumed as the carbon source than xylose (45%), because of the increased butyrate production, which inhibited xylose metabolism more. The carbon distributions in the products shifted to more butyrate (from 66.5% to 73.1%) and less acetate (from 17.8% to 0%) in the presence of BV. Adding acetate in the fermentation medium further

increased butyrate flux to ~76%, with the remaining substrate carbons mainly in cell biomass and CO₂, which was not monitored in this study. Clearly, adding the electronic carrier BV shifted the metabolic flux toward butyric acid biosynthesis, achieving complete butyrate selectivity against acetate with butyrate as the sole acid product in the fermentation. It should be noted that complete butyrate selectivity was also observed in *C. tyrobutyricum* BAS 7 by using a cathodic electron donor to alter NADH formation and flux distribution (Choi et al., 2012). However, only a low butyric acid concentration of 8.8 g/L was reached and the complicated electro-bioreactor system would be difficult to scale up for industrial application.

4. Discussion

Lignocellulosic biomass as a cost-effective feedstock for fermentation to produce chemicals and fuels requires efficient utilization of both glucose and xylose by microorganisms (Cho et al., 2015; Gu et al., 2014; Ho et al., 1998; Jaros et al., 2013; Liu et al., 2013; Wang et al., 2014). However, when multiple carbon sources are present, the consumption of the preferred carbon source (i.e., glucose) usually prevents the uptake and catabolism of the secondary substrates (e.g., xylose), a phenomenon called CCR (Görke and Stülke, 2008). The glucose-mediated CCR has also been observed in clostridia, including *C. acetobutylicum*, *C. beijerinckii*, and *C. tyrobutyricum* (Gu et al., 2010; Ounine et al., 1985; Xiao et al., 2012; Yu et al., 2015). To date, various metabolic engineering strategies have been used to address the inefficient xylose utilization problem caused by glucose repression in solventogenic *Clostridium*, including disrupting the global transcriptional regulator CcpA (Ren et al., 2010; Wu et al., 2015), the putative

xylose transcriptional repressor XylR (Hu et al., 2011; Xiao et al., 2012) and enzyme II in the glucose PTS (Xiao et al., 2011), and overexpressing key genes (*tal*, *tkl*, *rpi* and *rpe*) in the pentose phosphate pathway (Cu et al., 2009; Jin et al., 2014) and genes (*xylT*, *xylA*, and *xylB*) in the xylose catabolic pathway (Yu et al., 2015). Among them, overexpressing *xylT*, *xylA*, and *xylB* was able to greatly increase butanol production from glucose and xylose as co-substrates without CCR or any undesirable effects (Yu et al., 2015). Overexpressing *xylT*, *xylA*, and *xylB* also enabled *Propionibacterium freudenreichii* to utilize xylose in the presence of glucose without CCR (Wei et al., 2016). Clearly, the xylose utilization pathway is the prime target for metabolic engineering to overcome the CCR. Although this metabolic engineering strategy has been demonstrated for enhancing butanol and propionic acid production from xylose, it has not been used nor studied for enhancing butyric acid production from xylose previously.

In the present study, three genes encoding a D-xylose-proton symporter (*xylT*, CAC1345), an alternative xylose isomerase (*xylA*, CAC2620) and a xylulokinase (*xylB*, CAC2612) associated with xylose catabolism in *C. acetobutylicum* (Gu et al., 2010) were co-expressed in *C. tyrobutyricum*. The mutant Ct-pTBA overexpressing these heterologous genes under the control of native *thl* promoter was able to efficiently co-utilize glucose and xylose for butyric acid production at a significantly higher productivity (104%) and yield (14%) as compared to the parental wild-type strain (see Fig. 2). XylA and XylB were considered as the rate-limiting enzymes in the xylose catabolic pathway in clostridia (Xin et al., 2014), and their expressions were usually induced by xylose and repressed by glucose (Gu et al., 2010). However, these regulations should not affect the constitutive expressions of *C. acetobutylicum* XylT, XylA, and XylB and

1 their activities in *C. tyrobutyricum*, thus removing the CCR and enhancing xylose utilization by
2 Ct-pTBA. It is noted, however, that the xylose utilization efficiency or rate increased with
3 increasing the xylose concentration or xylose/glucose ratio and decreased with decreasing the
4 fermentation pH. Similar effects were also observed with glucose utilization, but the pH effect
5 was less pronounced. This concentration and pH dependent xylose utilization by clostridia has not
6 been reported previously.

7 Economical production of butyric acid from lignocellulose hydrolysates also requires high
8 final titer, productivity, yield, and purity (Zhu et al., 2002). *C. tyrobutyricum* produces butyrate
9 concomitantly with acetate, carbon dioxide, and hydrogen (see Fig. 6). Different metabolites are
10 produced to balance redox and provide ATP needed for cell growth and metabolism. In general,
11 attempts to knockout genes (*ack* and *pta*) in the acetate biosynthesis pathway failed to eliminate
12 acetate production (Lee et al., 2016; Liu et al., 2006; Zhu et al., 2005). Since metabolic flux
13 distribution is dictated by the available reducing power or NADH (Zhou et al., 2010), various
14 strategies have been used to increase NADH availability for the biosynthesis of the more reduced
15 product butyrate (vs. acetate) in butyric acid fermentation, including inactivating hydrogenase
16 (*hydA*) (Jang et al., 2014) and adding artificial electron carriers, such as MV, BV and neutral red
17 (NR), to divert the electrons from hydrogen to NADH production (Choi et al., 2012; Du et al.,
18 2014; Rao and Mutharasan, 1987; Reimann et al., 1996; Yu et al., 2015). Choi et al. (2012) showed
19 that MV addition increased intracellular NADH pool by ~3 times in *C. tyrobutyricum* BAS 7 with
20 greatly reduced hydrogen production and enhanced butyrate yield and selectivity. The main effect
21 of BV or MV addition is thus to increase the amount of NADH produced by the Ferredoxin-NAD⁺

reductase (see **Tables S1-S3** in Supplemental Materials), as was also previously demonstrated in *C. acetobutylicum* with MV or NR addition (Girbal et al., 1995; Peguin et al., 1994). In the present study, we demonstrated that BV addition at a low concentration of 0.75–3.75 μ M could effectively shift the flux for acetate from its formation to re-assimilation to butyrate, achieving a complete selectivity for butyrate as the sole acid product from glucose and xylose. Although a high butyrate selectivity could also be accomplished by knocking out competitive NADH-consuming pathways in engineered *E. coli* (Lim et al., 2013) and *C. acetobutylicum* (Jang et al., 2014), adding a small amount of BV without knocking out multiple genes in the genome, which often causes unhealthy cell growth and metabolism, is a simple and effective approach that can be easily scaled up for industrial application. It should also be noted that acetate re-assimilation can potentially increase the overall butyrate yield from sugar to 0.73 g/g since acetate can be produced at ~100% yield (g/g) from glucose in a homoacetic fermentation (Yang et al., 2013). Furthermore, adding acetate as a co-substrate with sugar also inhibited acetate production and increased butyrate production to a selectivity of ~1.0, which was also reported recently with an acetate-consuming *Clostridium* sp. S1 (Kim et al., 2016). However, xylose utilization by *Clostridium* sp. S1 was partially repressed by glucose due to CCR.

5. Conclusions

The glucose-mediated CCR was eliminated by overexpressing three heterologous xylose catabolism genes (*xylT*, *xylA* and *xlyB*) in *C. tyrobutyricum*. The engineered strain Ct-pTBA was capable of producing butyric acid from glucose and xylose simultaneously at high sugar utilization

rate and efficiency. The addition of BV as an artificial electron carrier facilitated the re-assimilation of acetate and increased butyrate production to a high final titer of 46.4 g/L, yield of 0.43 g/g sugar consumed, productivity of 0.87 g/L·h, and acid purity of 98.3% in free-cell batch fermentation, which were the highest ever reported for butyric acid fermentation. The engineered strain with BV addition can provide an economical process for butyric acid production from lignocellulosic biomass.

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Appendix A. Supplementary material

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Table 1

Strains, plasmids and primers used in this study.

Strain/Plasmid	Description	Source
Strains		
<i>E. coli</i> DH5 α	Host cells for plasmids amplification	Invitrogen
<i>E. coli</i> CA434	Donor cells for conjugation transformation	Williams et al., 1990
Ct	<i>C. tyrobutyricum</i> ATCC 25755	Liu et al., 2006
Ct(Δ ack) -pTBA	<i>adhE2</i> and <i>xylTBA</i> overexpression in Ct(Δ ack)	Yu et al., 2015
Ct-pTBA	Ct overexpressing <i>xylTBA</i>	This study
Plasmid		
pMTL82151	ColE1 ori; Cm ^R ; pBP1 ori, <i>TraJ</i>	Heap et al., 2009
pMAD22	From pMTL82151; P- <i>thl</i> :: <i>adhE2</i>	Yu et al., 2011
pTBA	From pMTL82151; P- <i>thl</i> :: <i>xylTBA</i>	This study
Primers		
	Sequence (5'-3')	
XylTB-for	ATTTAAATTTGGATCC AGATTGAGGAGGAATATAAAATGAATAA	
XylTB-rev	AAACAGCTATGACCGAGCTCGCGCTCCTACTTTTAACTATTTATATATCT	
XylA-for	AAAAGTAGGAGCGCGAGGAGGAATTAAAATGAATAATACACCAA	
XylA-rev	AAACAGCTATGACCGCCGCGGATTACTCAAAGGATTTTCTGTTTT	

Table 2

Fermentation kinetics of Ct and Ct-pTBA in CGM using glucose, xylose and glucose/xylose mixture (1:1) as substrates in serum bottles with 40 g/L CaCO₃

Strain	Substrate	Δ Glucose (g/L·h)	Δ Xylose (g/L·h)	Glucose consumed (%)	Xylose consumed (%)	Acetate (g/L)	Butyrate		
							Titer (g/L)	Yield (g/g)	Productivity (g/L·h)
Ct	Glucose	0.64±0.02	–	68.2±0.1	–	2.0±0.1	14.5±0.2	0.42±0.01	0.25±0.01
	Xylose	–	0.24±0.03	–	39.2±3.3	0.3±0.0	9.3±0.1	0.42±0.03	0.09±0.01
	Glucose/Xylose	0.54±0.03	0.02±0.02	100	4.9±2.5	1.7±0.1	9.5±0.3	0.35±0.01	0.20±0.04
Ct-pTBA	Glucose	0.46±0.02	–	72.4±2.3	–	1.6±0.1	15.9±0.1	0.42±0.01	0.16±0.04
	Xylose	–	0.32±0.03	–	46.4±1.1	0.3±0.0	10.6±0.2	0.39±0.02	0.10±0.02
	Glucose/Xylose	0.43±0.07	0.10±0.03	100	34.4±2.1	1.9±0.1	13.5±0.1	0.36±0.01	0.16±0.04

Δ Glucose: Glucose consumption rate; Δ Xylose: Xylose consumption rate

Table 3

Fermentation kinetics of Ct-pTBA grown on glucose, xylose and glucose/xylose mixtures in bioreactor

Glucose:Xylose ratio (g/g)	pH	μ (h ⁻¹)	Δ Glucose (g/L·h)	Δ Xylose (g/L·h)	Glucose consumed (%)	Xylose consumed (%)	Acetate (g/L)	Butyrate		
								Titer (g/L)	Yield (g/g)	Productivity (g/L·h)
1:0	6.0	0.16±0.01	2.79±0.20	–	100	–	8.0±0.4	36.5±0.3	0.30±0.02	0.86±0.06
0:1	6.0	0.09±0.01	–	1.78±0.08	–	91±1	5.9±0.4	36.2±0.2	0.30±0.02	0.51±0.05
1:3	6.0	0.11±0.01	0.60±0.05	1.80±0.12	100	96±0	8.2±0.5	39.4±0.4	0.30±0.02	0.65±0.06
3:1	6.0	0.14±0.01	1.80±0.16	0.73±0.06	100	95±2	8.7±0.5	37.4±0.4	0.32±0.02	0.84±0.06
1:1	5.0	0.14±0.01	0.66±0.02	0.38±0.02	58±1	29±1	5.3±0.5	15.6±0.4	0.29±0.01	0.34±0.03
1:1	5.5	0.14±0.01	1.11±0.03	0.97±0.01	100	66±2	8.7±0.3	30.2±0.4	0.29±0.01	0.55±0.04
1:1	6.0	0.14±0.01	1.20±0.04	1.40±0.12	100	94±1	8.6±0.6	37.8±0.4	0.32±0.02	0.86±0.10
1:1	6.0	0.13±0.01	1.16±0.05	1.14±0.11	100	84±1	0	39.8±0.4	0.36±0.02*	0.75±0.10
	(BV0.75)								0.42±0.01 ⁺	
1:1	6.0	0.13±0.01	1.27±0.05	0.86±0.10	100	77±1	0.8±0.1	46.4±0.4	0.43±0.02*	0.87±0.10
	(BV3.75)								0.52±0.01 ⁺	

 μ : Specific growth rate; Δ Glucose: Glucose consumption rate; Δ Xylose: Xylose consumption rate

* Overall yield for the entire batch fermentation

⁺ Butyrate yield for the period with BV addition

Table 4

Effects of benzyl viologen on flux distributions and carbon balances on substrate utilization, acetate re-assimilation and product formation in batch fermentations of Ct-pTBA grown on glucose and xylose at pH 6.0

	Substrates		Products			
	Glucose (%)	Xylose (%)	Acetate (%)	Butyrate (%)	Acetate (%)	Lactate (%)
Ct	87.8 ± 1.3	12.2 ± 1.3	0	56.7 ± 0.2	28.7 ± 1.0	0
Ct-pTBA	50.0 ± 0.7	50.0 ± 0.7	0	66.5 ± 0.5	17.8 ± 0.7	0
Ct-pTBA, BV 0.75 µM	55.0 ± 2.2	45.0 ± 2.2	0	73.1 ± 0.7	0	0.9 ± 0.2
Ct-pTBA, BV 3.75 µM	48.3 ± 1.3	37.6 ± 0.9	14.1 ± 0.4	76.0 ± 1.5	2.1 ± 0.3	0

Glucose:Xylose ratio in the medium was 1:1. Carbon balance is normalized with the initial total moles of substrate carbon as 100%.

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Figure 1. Glucose and xylose catabolism in *C. tyrobutyricum*. Xylose catabolism is repressed by glucose via a putative xylose transcriptional repressor XylR and the catabolite control protein A (CcpA). Genes in the pathway: *xylT*, D-xylose-proton symporter; *xylA*, xylose isomerase; *xylB*, xylulokinase; *tal*, transaldolase; *tkl*, transketolase; *rpi*, ribose-5-phosphate isomerase; *rpe*, ribulose-5-phosphate 3-epimerase; *glcG*, Enzyme II of the D-glucose phosphoenolpyruvate-dependent phosphotransferase system (PTS).

Figure 2. Fermentation kinetics of *C. tyrobutyricum* Ct and Ct-pTBA in CGM using glucose/xylose mixture (1:1) as substrates at pH 6.0 in bioreactor. Each fermentation condition was repeated at least twice and typical fermentation kinetics is shown here.

Figure 3. Fermentation kinetics of *C. tyrobutyricum* Ct-pTBA in CGM using glucose/xylose mixture (1:1) as substrates at various pH values in bioreactor. Each fermentation condition was repeated at least twice and typical fermentation kinetics is shown here.

Figure 4. Fermentation kinetics of *C. tyrobutyricum* Ct-pTBA in CGM using glucose, xylose, and glucose/xylose mixture (3:1 and 1:3), respectively, as carbon sources at pH 6.0 in bioreactor. Each fermentation condition was repeated at least twice and typical fermentation kinetics is shown here.

Figure 5. Fermentation kinetics of *C. tyrobutyricum* Ct-pTBA in CGM with glucose/xylose mixture (1:1) as substrates and BV as an artificial electron carrier at pH 6.0 in bioreactor. (A) 0.75 μ M BV added at ~37 h; (B) 3.75 μ M BV with 18 g/L acetate added at ~48 h. Each fermentation condition was repeated at least twice and typical fermentation kinetics is shown here.

Figure 6. Metabolic pathways for butyrate, acetate, lactate and hydrogen production from glucose and xylose in *C. tyrobutyricum*. Butyrate biosynthesis is mainly via a CoA transferase (CoAT), which transfers the CoA from butyryl-CoA to acetate. Benzyl viologen (BV) can divert electrons from hydrogen production to NADH generation, which enhances

butyrate biosynthesis and acetate re-assimilation, and may cause lactate accumulation (indicated in red dashed line) due to excessive NADH accumulation. Enzymes involved in the pathway: lactate dehydrogenase (LDH), hydrogenase (HydA), phosphotransacetylase (PTA), acetate kinase (AK), phosphotransbutyrylase (PTB), butyrate kinase (BK), CoA transferase (CoAT).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6