Cellular and Metabolic Engineering **Biotechnology and Bioengineering**

DOI 10.1002/bit.25613

Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production through co-utilization of glucose and xylose†

Le Yu[1], Mengmeng Xu[1], I-Ching Tang[2], and Shang-Tian Yang[1,*]

[1] William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, 151 West Woodruff Ave., Columbus, Ohio 43210, USA

[2] Bioprocessing Innovative Company, 4734 Bridle Path Ct., Dublin, OH 43017, USA

Running Title: Metabolic engineering of *C. tyrobutyricum* for butanol production

*Corresponding author. Phone: 614-292-6611; Fax: 614-292-3769; Email: yang.15@osu.edu

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.25613]

Additional Supporting Information may be found in the online version of this article.

Received February 27, 2015; Revision Received April 8, 2015; Accepted April 9, 2015

Abstract

The glucose-mediated carbon catabolite repression (CCR) in *C. tyrobutyricum* impedes efficient utilization of xylose present in lignocellulosic biomass hydrolysates. In order to relieve the CCR and enhance xylose utilization, three genes (*xylT*, *xylA*, and *xylB*) encoding a xylose proton-symporter, a xylose isomerase and a xylulokinase, respectively, from *C. acetobutylicum* ATCC 824 were co-overexpressed with aldehyde/alcohol dehydrogenase (*adhE2*) in *C. tyrobutyricum* (Δ*ack*,). Compared to the strain Ct(Δ*ack*)-pM2 expressing only *adhE2*, the mutant Ct(Δ*ack*)-pTBA had a higher xylose uptake rate and was able to simultaneously consume glucose and xylose at comparable rates for butanol production. Ct(Δ*ack*)-pTBA produced more butanol (12.0 vs. 3.2 g/L) with a higher butanol yield (0.12 vs. 0.07 g/g) and productivity (0.17 vs. 0.07 g/L·h) from both glucose and xylose, while Ct(Δ*ack*)-pM2 consumed little xylose in the fermentation. The results confirmed that the CCR in *C. tyrobutyricum* could be overcome through overexpressing *xylT*, *xylA*, and *xylB*. The mutant was also able to co-utilize glucose and xylose present in soybean hull hydrolysate (SHH) for butanol production, achieving a high butanol titer of 15.7 g/L, butanol yield of 0.24 g/g and productivity of 0.29 g/L·h. This study demonstrated the potential application of Ct(Δ*ack*)-pTBA for industrial biobutanol production from lignocellulosic biomass.

Keywords: butanol; carbon catabolite repression; *Clostridium tyrobutyricum*; soybean hull hydrolysate; xylose; metabolic engineering

Introduction

Biological production of n-butanol, an important industrial solvent and potentially an advanced biofuel, from lignocellulosic biomass in acetone-butanol-ethanol (ABE) fermentation by solvent-producing *Clostridium acetobutylicum* and *Clostridium beijerinckii* has drawn increasing attentions in recent years (Green, 2011; Jang et al., 2012; Wang et al., 2014). However, conventional ABE fermentation with the biphasic metabolic shift from acidogenesis to solventogenesis is difficult to control and is prone to "acid crash" due to the complex metabolic regulation in solventogenic *Clostridium* (Zhao et al., 2013; Xu et al., 2015). Recently, we have engineered *Clostridium tyrobutyricum*, an acidogen natively producing acetate and butyrate as two major fermentation products from glucose and xylose (Liu et al., 2006), to produce n-butanol by overexpressing *adhE2* encoding a bifunctional aldehyde/alcohol dehydrogenase (Yu et al., 2011). The engineered strain *C. tyrobutyricum* (Δ*ack*, *adhE2*) produced butanol, along with butyrate and acetate, throughout the fermentation. Besides being stable without subjecting to biphasic metabolic shift, higher butanol tolerance and lack of acetone-synthetic pathway in *C. tyrobutyricum* are also beneficial to butanol biosynthesis and downstream purification (Yu et al., 2012). Furthermore, butanol became the main fermentation product by *C. tyrobutyricum* (Δ*ack*, *adhE2*) in the presence of methyl viologen (MV), an artificial electron carrier, which increased NADH availability and resulted in a high butanol titer and yield (Du et al., 2015).

Lignocellulosic biomass is the most abundant renewable resource, and its utilization as the fermentation substrate is a key to economically viable biobutanol production (Kumar et al.,

2013). However, a major problem of fermenting lignocellulosic biomass hydrolysates by most of clostridia, including *C. acetobutylicum*, *C. beijerinckii* and *C. tyrobutyricum*, is the inefficient co-utilization of glucose and xylose, the two major sugars present in lignocellulose. Although these clostridia can use xylose as the sole carbon source, negligible or limited amount of xylose was consumed in the presence of glucose, leading to incomplete substrate utilization and low solvent production (Ounine et al., 1985; Xiao et al., 2012). The inhibited xylose metabolism is caused by the glucose-mediated carbon catabolite repression (CCR), a common phenomenon observed in many microbes (Aristidou and Penttila, 2000; Görke and Stülke, 2008; Yao and Shimizu, 2013). Various efforts have been made to overcome the CCR in *C. acetobutylicum* (Gu et al., 2009; Hu et al., 2011; Jin et al., 2014; Ren et al., 2010; Xiao et al., 2011; Wu et al., 2015). It has been suggested that the inherent rate-limiting steps of xylose utilization in *Clostridium* occur in the metabolic pathway prior to the pentose phosphate pathway (PPP) due to lack of an efficient xylose transport system as well as strong effects of glucose repression on these metabolic reactions (Grimmler et al., 2010; Gu et al., 2010; Xiao et al., 2011; 2012).

In this work, we studied the feasibility of engineering *C. tyrobutyricum* to simultaneously and efficiently use a mixture of glucose and xylose for n-butanol production. Although the draft genome of *C. tyrobutyricum* ATCC 25755 contains genes encoding a sugar:proton symporter with a high amino acid identity with the xylose symporter (*xylT*) from *C. acetobutylicum* ATCC 824, and putative xylose isomerase and xylulokinase, these genes have not been experimentally verified and may also be subjected to glucose CCR. We thus speculated that the bottleneck in xylose utilization, involving xylose transport, isomerization, and xylulose phosphorylation as

observed in solventogenic *Clostridium* (Gu et al., 2010; Xiao et al., 2011; 2012), also exists in *C. tyrobutyricum*. Therefore, to facilitate xylose transport and metabolism in *C. tyrobutyricum*, three genes, encoding a xylose proton-symporter (*xylT*, CAC1345), a xylose isomerase (*xylA*, CAC2610), and a xylulokinase (*xylB*, CAC2612), respectively, from *C. acetobutylicum* ATCC 824 were constitutively co-expressed with *adhE2* in *C. tyrobutyricum* (Δ*ack*). Co-utilization of glucose and xylose with significantly reduced residual xylose was achieved in the mutant Ct(Δ*ack*)-pTBA, resulting in greatly enhanced butanol production. The co-fermentation of glucose and xylose showed comparable cell growth and butanol production with all xylose efficiently consumed at all glucose-to-xylose ratios studied, confirming that the CCR bottleneck in the fermentation of glucose-xylose mixture by *C. tyrobutyricum* could be effectively alleviated by enhancing the xylose metabolic pathway. Finally, efficient utilization of glucose and xylose present in soybean hull hydrolysate (SHH) by the mutant was demonstrated.

Materials and Methods

Bacterial strains, plasmids, and culture media

The bacterial strains and recombinant plasmids used in this study are listed in **Table I**. *C. tyrobutyricum* ATCC 25755 with *ack* knockout (Liu et al., 2006) and all mutant strains derived from it were cultured in Clostridial Growth Medium (CGM) containing 4 g/L Tryptone, 2 g/L yeast extract, 1 g/L $K_2HPO_4 \cdot 3H_2O$, 0.5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, trace minerals (Zhu and Yang, 2003), and glucose or xylose as carbon source, unless otherwise noted, at 37 °C under anaerobic conditions. *E. coli* strains used in this study were grown aerobically at 37 °C in Luria-Bertani (LB) medium or on LB agar plates. All media were

autoclaved at 121 ^{o}C for 30 min, and supplemented with appropriate antibiotics (25 μg/ml chloramphenicol, 45 μg/ml thiamphenicol, or 250 μg/ml cycloserine) after autoclaving.

Plasmids construction

The plasmid pTBA (see **Figure S1** in supplementary materials) was constructed from pMTL82151-*adhE2* (pM2) (Yu et al., 2011) by sequentially inserting *xylT* (CAC1345), *xylB* (CAC2612), and *xylA* (CAC2610), which were amplified from *C. acetobutylicum* ATCC 824 genomic DNA by PCR using the primers shown in **Table I**, after *adhE2* at the *Sac*II cutting site using the Clontech infusion cloning kit (Clontech Laboratories, Inc., Mountain View, CA). The constitutive co-expressions of the four genes were driven by the native thiolase (*thl*) promoter from *C. tyrobutyricum* (Yu et al., 2011), and the original ribosome binding site of each gene was replaced with the consensus sequence "AGGAGG" to optimize the expression.

Transformation

The plasmid pTBA was transformed into *C. tyrobutyricum* (Δ*ack*) by conjugation (Yu et al. 2012). *E. coli* CA434 carrying the plasmids to be transformed were cultivated in LB medium containing 25 μg/ml chloramphenicol at 37°C overnight to reach OD_{600} of 1.5–2.0. The donor cells were collected by centrifugation at 4,000×g for 2 min, washed once using 1 ml sterile phosphate-buffered saline (PBS), mixed with 200 μl of *C. tyrobutyricum* cells precultured at 37 °C overnight, and the mixture was pipetted onto CGM agar plates. After incubating in an anaerobic chamber at 37 °C for 8–12 h, cells were collected and re-suspended in 1 ml of PBS and spread onto CGM plates containing 45 μg/ml thiamphenicol and 250 μg/ml cycloserine. The plates were incubated for 2–3 days to obtain colonies, and positive transformants, which were

confirmed by colony PCR screening and plasmid extraction, were selected and stored at -80 °C.

Hydrolysis of soybean hull and detoxification

Soybean hull, obtained from Minnesota Soybean Processors, was hydrolyzed to release glucose and xylose by dilute acid pretreatment and enzymatic hydrolysis according to a previously optimized method (Dong et al., 2014). Briefly, 100 g soybean hull particles were suspended in 1 L of 0.04 N HCl solution, followed by autoclaving at 121 °C, 15 psig for 30 min. The solution was then adjusted to pH 5.0 using sodium hydroxide and treated with 3.0 g Cellic CTec2 (containing cellulases, β-glucosidases, and hemicullulase; Novozymes North America, Inc., Franklinton, NC) for 72 h at 50 °C. After removing the undissolved solids by centrifugation at 7000 rpm for 15 min, the hydrolysate was concentrated three times by vacuum rotary evaporation at 60 °C. The concentrated hydrolysate was then adjusted to pH 2.0 with HCl and detoxified with 2% (w/w) activated carbons at 80 °C in a water bath for 60 min. After removing activated carbons by centrifugation, the soybean hull hydrolysate (SHH), containing ~45 g/L glucose and ~20 g/L xylose, was adjusted to pH 6.5 with sodium hydroxide and stored at 4 °C. Before use in batch fermentation kinetics studies, SHH was supplemented with additional xylose to bring the glucose/xylose ratio to ~1.0 and the total sugar concentration to ~90 g/L in the fermentation medium so that butanol production would not be limited by the availability of sugar.

Fermentation kinetics

Batch fermentation kinetics was studied in serum bottles containing 50 ml CGM and 1-L stirred-tank bioreactor containing 600 ml of CGM or SHH. The bioreactor was autoclaved at 121

^{o}C for 30 min and then sparged with nitrogen for ~30 min to reach anaerobiosis, while the serum bottles were sparged with nitrogen first before autoclaving. An overnight culture was used to inoculate the reactor at a volume ratio of 5%. Thiamphenicol (45 μg/ml) was also added at the time of inoculation to ensure plasmid stability during the fermentation. The pH in the bioreactor was controlled at 6.0 with 40% ammonium hydroxide and samples were collected twice a day at regular intervals. For fermentation in serum bottles, samples (~1 ml each) were taken daily, with pH adjusted manually to 6.3–6.5 by adding 10% sodium hydroxide drop by drop with a syringe and gas released to avoid pressure build-up. All fermentations were repeated at least once and representative data with averages and standard errors are reported.

Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). Glucose and xylose were analyzed using a high performance liquid chromatograph (HPLC) equipped with an organic acid analysis column (Bio-Rad HPX-87H) and a refractive index detector (Shimadzu RID-10A) at 45 °C (Yu et al., 2011). Acetone, butanol, ethanol, acetate and butyrate were determined with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30 m fused silica column (0.25 m film thickness and 0.25 mm ID, Stabilwax-DA) following the method described elsewhere (Jiang et al., 2014).

Statistical analysis

Unless otherwise noted, batch fermentation for each condition was repeated once, and product concentrations, yields and productivities are reported as average with standard errors.

Student's *t*-test analysis was performed using JMP software with the significance level $\alpha = 0.05$.

Results

Comparison of Ct(Δack)-pTBA and Ct(Δack)-pM2 in xylose utilization

Xylose metabolism in *C. tyrobutyricum* Ct(Δ*ack*)-pTBA overexpressing the three genes, *xylT*, *xylB* and *xylA*, responsible for xylose metabolism in *C. acetobutylicum* ATCC 824 was first evaluated and compared with Ct(Δ*ack*)-pM2 in batch fermentations using xylose as the sole carbon source. As shown in **Figure 1**, Ct(Δ*ack*)-pTBA had a longer lag phase (~24 h vs. ~10 h for the control), probably the result of the extra metabolic burden in overexpressing *xylTBA*, but a higher xylose consumption rate (2.6 g/L·h vs. 1.7 g/L·h) in the exponential phase, apparently because of the overexpression of *xylTBA*. However, both strains had similar specific growth rate and butanol production, with butanol titer, yield and productivity reaching 8.6 g/L, 0.09 g/g and 0.14 g/L·h, respectively.

Improved co-utilization of glucose and xylose by Ct(Δack)-pTBA

To evaluate the performance of xylose catabolism in the presence of glucose, batch fermentations of glucose and xylose mixture at pH 6.0 were carried out for Ct(Δ*ack*)-pTBA and Ct(Δ*ack*)-pM2, and the results are shown in **Figure 2** and **Table II**. As expected, glucose and xylose were co-utilized by the mutant in the log phase, at comparable rates of 1.03 g/L·h for glucose and 1.05 g/L·h for xylose. In contrast, the control strain could uptake glucose at a higher rate (1.85 g/L·h) but with negligible xylose consumption even after the depletion of glucose. Clearly, the control strain was subjected to catabolic repression in xylose metabolism. It should be noted that the mutant showed a slight delay in using xylose, compared to its utilization of

glucose, which might be caused by the extra metabolic burden from the *xylTBA* overexpression as mentioned before. The delayed utilization of xylose was a common phenomenon also observed in other studies (Gu et al., 2009; Ren et al., 2010; Xiao et al., 2011), suggesting that the initial steps in xylose catabolism (such as xylose transport) might be crucial in glucose-mediated CCR. Overall, xylose utilization by the mutant was greatly improved with a total consumption of 87.3% of xylose, whereas only limited amount of xylose (10.6%) was consumed by the control, which occurred after glucose was nearly depleted. Finally, butanol and ethanol production by the mutant increased to 12.0 g/L and 3.6 g/L, an improvement of 275% and 164%, respectively. Meanwhile, a higher butanol yield and productivity (0.12 g/g vs. 0.07 g/g, and 0.17 g/L·h vs. 0.07 g/L·h, respectively) were also obtained.

Xylose utilization by Ct(Δack)-pTBA in media with varying glucose-to-xylose ratios

In order to demonstrate the capability of Ct(Δ*ack*)-pTBA in glucose and xylose co-catabolism at different xylose concentrations, fermentations with different glucose-to-xylose ratios (3:1, 2:1, 1:1 and 1:2) were studied in serum bottles, and the results are shown in **Figure 3** and **Table III**. In general, co-utilization of glucose and xylose was observed at all tested ratios, all of which also gave similar specific growth rates and butanol and acid production. However, as the ratio of xylose increased, xylose uptake rate also increased from 0.14 g/L·h to 0.45 g/L·h, suggesting that the xylose consumption rate was closely related to the level of xylose. This phenomenon was also observed in previous studies with solventogenic *Clostridium* (Ren et al., 2010; Xin et al., 2014). Since xylose uptake by the xylose-proton symporter is a facilitated diffusion process driven by an electrochemical gradient (Cook et al., 2006), xylose transport rate

would depend on not only the expression level of the xylose symporter, but also the extracellular xylose concentration. On the contrary, the glucose uptake rate exhibited only a slight decrease as the ratio of glucose decreased, suggesting that ATP-dependent glucose transport systems might exist in *C. tyrobutyricum*, which could transport glucose without being affected by its extracellular level. Nevertheless, the data confirmed that the ability of active co-catabolism of glucose and xylose can be obtained even at a low extracellular xylose level, which has also been reported for two other *Clostridium* strains (Ren et al., 2010; Xin et al., 2014).

Fermentation of glucose and xylose in soybean hull hydrolysate

Figure 4 shows batch fermentation kinetics with SSH as substrate by Ct(Δ*ack*)-pTBA and Ct(Δ*ack*)-pM2, and the fermentation performances are also summarized in **Table II**. Similar to the fermentations with CGM, co-utilization of glucose and xylose, at the uptake rate of 1.20 g/L·h and 1.21 g/L·h, respectively, in SHH was observed for the mutant during the exponential phase, and 92.7% of xylose in the medium was consumed, whereas only 17.8% of xylose was consumed by the control. Consequently, the mutant produced much more butanol compared to the control since both sugars were used in the fermentation. Finally, 8.1 g/L of butanol was produced with the butanol yield of 0.10 g/g by the mutant. Meanwhile, more acids (~29 g/L) were also produced by the mutant, compared to ~16 g/L by the control.

To decrease acids production and direct more metabolic flux towards butanol biosynthesis, 500 μM methyl viologen (MV) was added at the beginning of the fermentation. With MV, butanol production increased to 15.7 g/L, while butyrate and acetate production decreased greatly to 5.8 g/L and 2.0 g/L, respectively. The decline in acids production also contributed to

improved butanol yield (0.24 g/g) and productivity (0.29 g/L·h). MV, as an artificial electron carrier, directed the electron towards NADH synthesis, instead of hydrogen production, and thus favored butanol biosynthesis, which required NADH (Du et al., 2015). However, the addition of MV showed negative effects on cell growth and xylose utilization, causing incomplete xylose consumption (65.2%) and decreased xylose uptake rate (0.76 g/L·h), probably because the expression and activities of XylABT were compromised by the reduced ATP as a result of less acids production. A previous study with glucose or xylose as the sole carbon source also showed that MV inhibited cell growth, decreased sugar uptake, and caused incomplete sugar consumption in the fermentation by Ct(Δ*ack*)-pM2 (Du, 2013). Compared to glucose, the negative effect of MV on xylose utilization was more severe because less ATP is generated in xylose metabolism. In addition, the higher butanol concentration might have also negatively affected xylose transport. Nevertheless, the production of 15.7 g/L butanol from SHH by the mutant was much higher than those previously obtained with *C. acetobutylicum* (7.6 g/L) and *C. beijerinckii* (10.0 g/L) (Dong et al., 2014).

It should be noted that similar performances of the glucose-xylose co-fermentation were obtained with CGM and SHH. One major obstacle in using lignocellulosic biomass in ABE fermentation is the presence of inhibitors generated from the pretreatment of lignocellulosic biomass, which would impair cell activity and solvent production (Baral and Shah, 2014). However, after treating with activated carbon, the detoxified SHH hydrolysate did not show any inhibition effect on *C. tyrobutyricum*. Activated carbon adsorption has also been used to detoxify soybean hull, sugarcane bagasse, cotton stalk, and wood pulping hydrolysates for ABE

fermentation (Dong, 2014; Dong et al., 2014; Lu et al., 2013). The efficient utilization of soybean hull hydrolysate as substrate by *C. tyrobutyricum* further demonstrated its potential application for biobutanol production from lignocellulosic biomass.

Discussion

In recent years, extensive studies have focused on ABE fermentation for butanol production from lignocellulosic biomass, including barley straw, corn stover, corn fiber, soybean hull, cassava bagasse, wheat bran, wheat straw, and wood pulp (Guo et al., 2013; Liu et al., 2006; Lu et al., 2012; 2013; Qureshi et al., 2008; 2010a; 2010b; Wang and Chen, 2011). Almost all of the previous studies have indicated the difficulty or slowness in the utilization of xylose present in lignocellulosic biomass hydrolysates, suggesting that the CCR for xylose catabolism is widely present in the ABE fermentation of lignocellulose. How to overcome the glucose-induced catabolic repression causing inefficient utilization of xylose and other sugars present in the biomass is a major challenge in using lignocellulose for biobutanol production. Although numerous attempts have been made in *C. acetobutylicum* and *C. beijerinckii* to improve the strains' ability in xylose catabolism, results to date are still far from ideal, especially with respect to simultaneous utilization of glucose and xylose.

CCR occurs when a preferred sugar (glucose in this study) is present in the medium, which negatively affects the metabolism of other sugars (xylose) through the repression mainly at the transcriptional level. Many efforts have been made to relieve the catabolite repression and to realize co-utilization of glucose and xylose in solventogenic *Clostridium*. The disruption of the catabolite control protein A (CcpA), which is the global transcriptional regulator in CCR (Lorca

et al., 2005; Moreno et al., 2004), in *C. acetobutylicum* improved xylose metabolism and butanol titer to ~12 g/L. However, the disruption of CcpA also caused undesirable changes in other metabolic activities because of impaired expression of CoA transferase and glycolytic genes (Ren et al., 2010). Although inserting a mutated *ccpA*^{V302N} into the chromosome of CcpA-disrupted *C. acetobutylicum* mutant improved glucose and xylose co-utilization, it led to much slower glucose consumption and solvents production (Wu et al., 2015). The disruption of *xylR* encoding a putative xylose transcriptional repressor XylR, which could repress *xylA* and *xylB* genes (Rodionov et al., 2001), improved xylose utilization and the final butanol titer to 11.4 g/L in *C. beijerinckii*, but not in *C. acetobutylicum* (Hu et al., 2011; Xiao et al., 2012). The *xylR* gene has not been identified in *C. tyrobutyricum* genome. The glucose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) also plays an important role in the induction of CCR (Tangney and Mitchell, 2007). However, the disruption of the gene (*glcG*) encoding an enzyme II in the glucose PTS in *C. acetobutylicum* did not significantly improve xylose consumption, suggesting that the subdued glucose PTS might not be sufficient to eliminate the CCR in *C. acetobutylicum* (Xiao et al., 2011).

To identify the rate-limiting steps in xylose catabolism, the effect of overexpressing genes in the pentose phosphate pathway (PPP) has also been investigated (Jin et al., 2014; Gu et al., 2009). However, the improvement in xylose utilization was limited and no evidence on glucose and xylose co-utilization was provided, indicating that the PPP might not be a major barrier in xylose catabolism. For xylose transport and metabolism before the PPP, two operons (CAC1344-1349 and CAC2610-2612) were identified in *C. acetobutylicum*, including one xylose symporter

(CAC1345), one xylose isomerase (CAC1346), one L-fucose isomerase (CAC2610, putative xylose isomerase, *xylA*), and two xylulokinases (*xylB*, CAC1344 and 2612) (Grimmler et al., 2010). Putative catabolite responsive element (CRE) sequences and xylose regulator (XylR) binding sites were identified in these two operons (Rodionov et al., 2001) and the transcriptional analysis indicated that multiple genes within the operons were subjected to catabolic repression, except for *xylA* (CAC2610) and *xylB* (CAC2612), which have been proven to be indispensible in xylose catabolism by comparative genomics analysis (Grimmler et al., 2010; Gu et al., 2010). The importance of these two enzymes in xylose catabolism was also confirmed in a novel *Clostridium* strain BOH3 with much higher xylose isomerase and xylulokinase activities, which might contribute to its exceptional capability of co-utilizing glucose and xylose, as compared to other solventogenic *Clostridium* (Xin et al., 2014).

Previous studies have identified the putative rate-limiting steps in xylose catabolism in *C. acetobutylicum*, including the transport and isomerization of xylose, and the phosphorylation of xylulose into xylulose-5-phosphate (Xiao et al., 2011; 2012). These steps were catabolite repressed mainly at the transcriptional level, and were also inherently inefficient (Grimmler et al., 2010; Xiao et al., 2011). Although overexpressing CAC1345, CAC2610, and CAC2612 individually or together led to enhanced xylose catabolism in the glucose/xylose co-fermentation by *C. acetobutylicum*, most of xylose was consumed only after glucose had been depleted.

In the present study, CAC1345, CAC2612, and CAC2610 (*xylTBA*) were co-expressed under the constitutive promoter *thl* in *C. tyrobutyricum.* As shown in the fermentation kinetics study, the heterologous expression of these genes facilitated efficient and complete co-utilization

of glucose and xylose, at comparable rates, without disrupting the glucose PTS or other regulators such as CcpA and XylR. This study provides the evidence that an improved xylose transport, isomerization, and phosphorylation process could accelerate xylose uptake and co-utilization with glucose, without the negative effect of CCR, in *C. tyrobutyricum.*

Conclusions

Three genes, *xylT*, *xylA*, and *xylB*, catalyzing the transport of xylose and the first two metabolic steps before the pentose phosphate pathway from *C. acetobutylicum* were co-overexpressed in *C. tyrobutyricum*, resulting in alleviated catabolite repression, efficient simultaneous consumption of glucose and xylose, and significantly enhanced butanol production, confirming the essential roles of *xylT*, *xylA*, and *xylB* in xylose metabolism. The fermentation kinetics with different glucose-to-xylose ratios demonstrated that efficient xylose consumption could be achieved even at low xylose levels. The fermentation of soybean hull hydrolysate by the mutant further confirmed its capability of utilizing cheap and abundant lignocellulosic biomass as feedstock. The mutant thus should have good potential for large-scale and cost-effective industrial biobutanol production. The results from this study also provide better understanding of xylose metabolism and glucose-mediated catabolite repression in *C. tyrobutyricum.*

Acknowledgements

This work was supported in part by the National Science Foundation STTR program (IIP-1026648).

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Table I. Bacterial strains and plasmids.

Strain/Plasmid	Relevant Characteristics	Reference / Source
Strains		
E. coli DH5α	Host cells for plasmids amplification	Invitrogen
E. coli CA434	Donor cells for conjugation transformation	Williams et al., 1990
Ct(Δ*ack*)	*C. tyrobutyricum* ATCC 25755 with *ack* knockout	Liu et al., 2008
Ct(Δ*ack*)-pM2	*adhE2* overexpression in Ct(Δ*ack*)	Yu et al., 2011
Ct(Δ*ack*)-pTBA	*adhE2* and *xylTBA* overexpression in Ct(Δ*ack*)	This study
Plasmids		
pMTL82151	ColE1 ori; Cm^r; pBP1 ori, *TraJ*	Heap et al., 2009
pM2	From pMTL82151; P-*thl adhE2*	Yu et al., 2011
pTBA	From pMTL82151; P-*thl adhE2 xylTBA*	This study
Primers	Sequence (5'-3')	
XylT-for	TTTGCTTCATTATCC CAGATTGAGGAGGAATATAAAATGAATAA	
XylT-rev	GAAACAGCTATGACC GAGCTC AACTACTCATTTAATCCTCTAACTTTTCCA	
XylB-for	TTAAATGAGTAGTTG AGGAGGTTTGATTATGAGGTATTTATTAGGTATAGAC	
XylB-rev	AAACAGCTATGACC GAGCTC GCGCTCCTACTTTTAACTATTTATATATCT	
XylA-for	AAAAGTAGGAGCGCG AGGAGGAATTAAAATGAATAATACACCAA	
XylA-rev	AAACAGCTATGACCG TCTCTTTTATTACTCAAAAGGATTTTCTG	

Table II. Kinetics of co-fermentation of glucose and xylose in CGM and SHH by Ct(Δ*ack*)-pM2 and Ct(Δ*ack*)-pTBA.

Strains	Medium	Sp. growth rate (h^{-1})	Glucose uptake rate (g/L·h)	Xylose uptake rate (g/L·h)	Xylose consumption (%)	Butanol			Butyrate (g/L)	Acetate (g/L)
						Titer (g/L)	Yield (g/g)	Productivity (g/L·h)		
Ct(Δ*ack*)-pM2	CGM	0.22±0.01	1.85±0.10	0	10.6±0.6	3.2±0.2	0.07±0.01	0.07±0.01	10.4±0.4	7.0±0.3
	SHH	0.21±0.01	1.43±0.03	0	17.8±1.0	3.5±0.1	0.06±0.01	0.10±0.01	10.3±0.5	5.8±0.8
Ct(Δ*ack*)-pTBA	CGM	0.21±0.00	1.03±0.07	1.05±0.08	87.3± 1.0	12.0±0.2	0.12±0.00	0.17±0.01	8.0±0.4	5.1±0.2
	SHH	0.20±0.01	1.20±0.11	1.21±0.08	92.7±0.8	8.1±0.1	0.10±0.01	0.15±0.01	20.1±0.6	8.7±0.5
	SHH+MV	0.16±0.01	1.29±0.12	0.75±0.07	65.2±1.3	15.7±0.4	0.24±0.02	0.29±0.01	5.8±0.3	2.0±0.5

CGM: Clostridial growth medium containing 45 g/L glucose and 60 g/L xylose.

SHH: soybean hull hydrolysate containing 45 g/L glucose and 45 g/L xylose.

Data shown are mean ± s.d. (n = 2).

Table III. Fermentation kinetics of Ct(Δ*ack*)-pTBA grown on glucose-xylose mixtures at different ratios in serum bottles.

Glucose to xylose ratio	Sp. growth rate (h^{-1})	Glucose uptake rate (g/L·h)	Xylose uptake rate (g/L·h)	Butanol productivity (g/L·h)	Butanol (g/L)	Butyrate (g/L)	Acetate (g/L)
3:1	0.13±0.02	0.53±0.03	0.14±0.01	0.05±0.01	8.5±0.8	8.1±1.1	5.4±0.4
2:1	0.14±0.02	0.53±0.02	0.19±0.02	0.05±0.00	8.5±0.6	7.9±0.6	5.5±0.6
1:1	0.13±0.03	0.50±0.04	0.30±0.01	0.05±0.01	8.2±0.6	8.5±0.5	5.2±0.6
1:2	0.13±0.03	0.45±0.02	0.45±0.02	0.05±0.01	8.1±0.7	8.9±0.4	5.5±0.8

Total initial sugar concentration: 60 g/L.

Data shown are mean ± s.d. (n = 2).

List of Figures

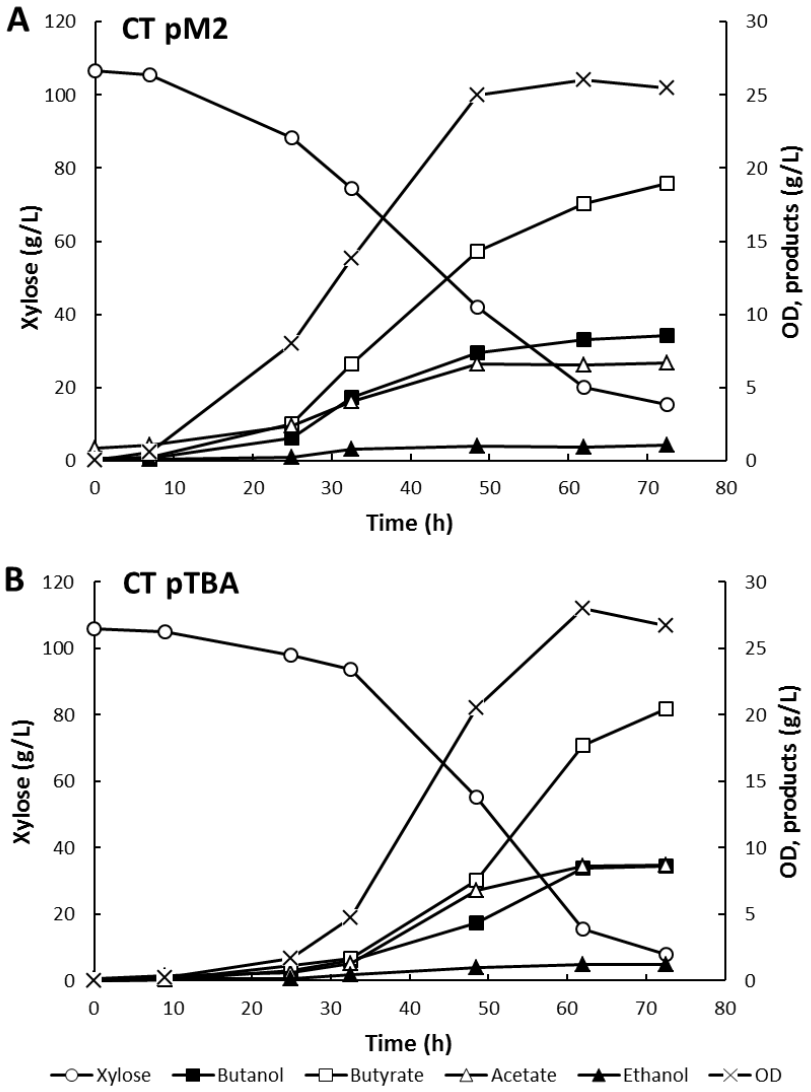

Figure 1.

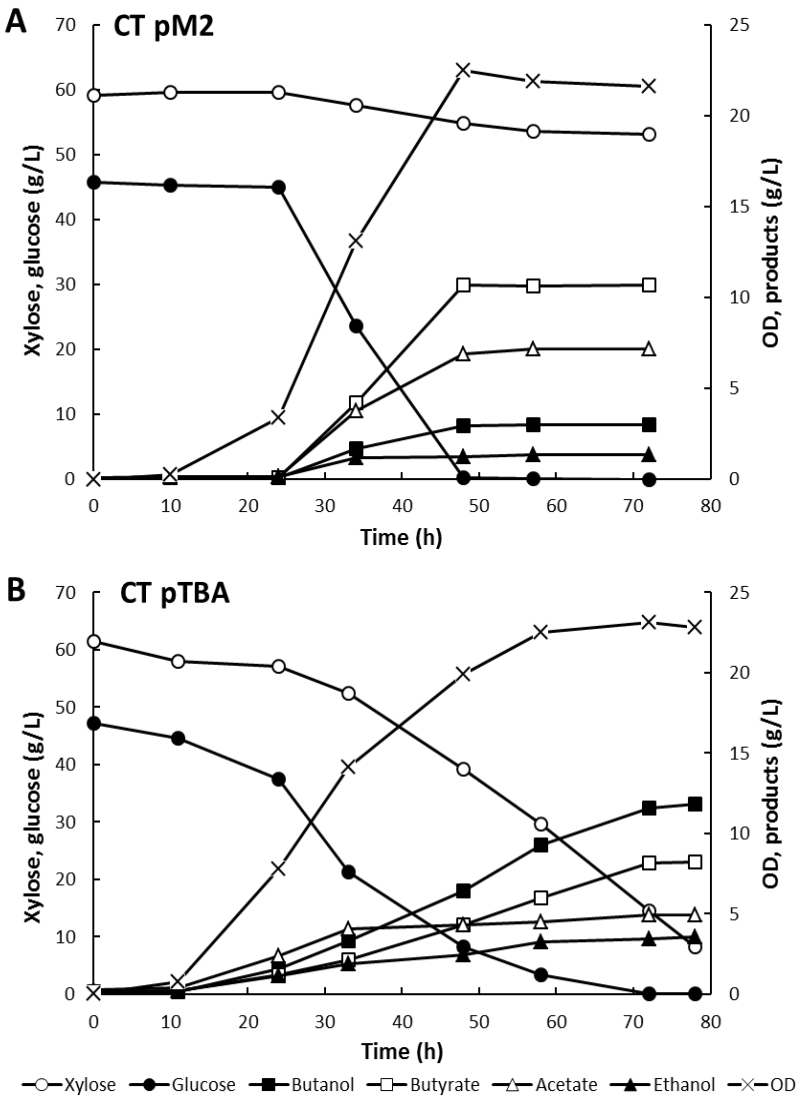

Figure 2.

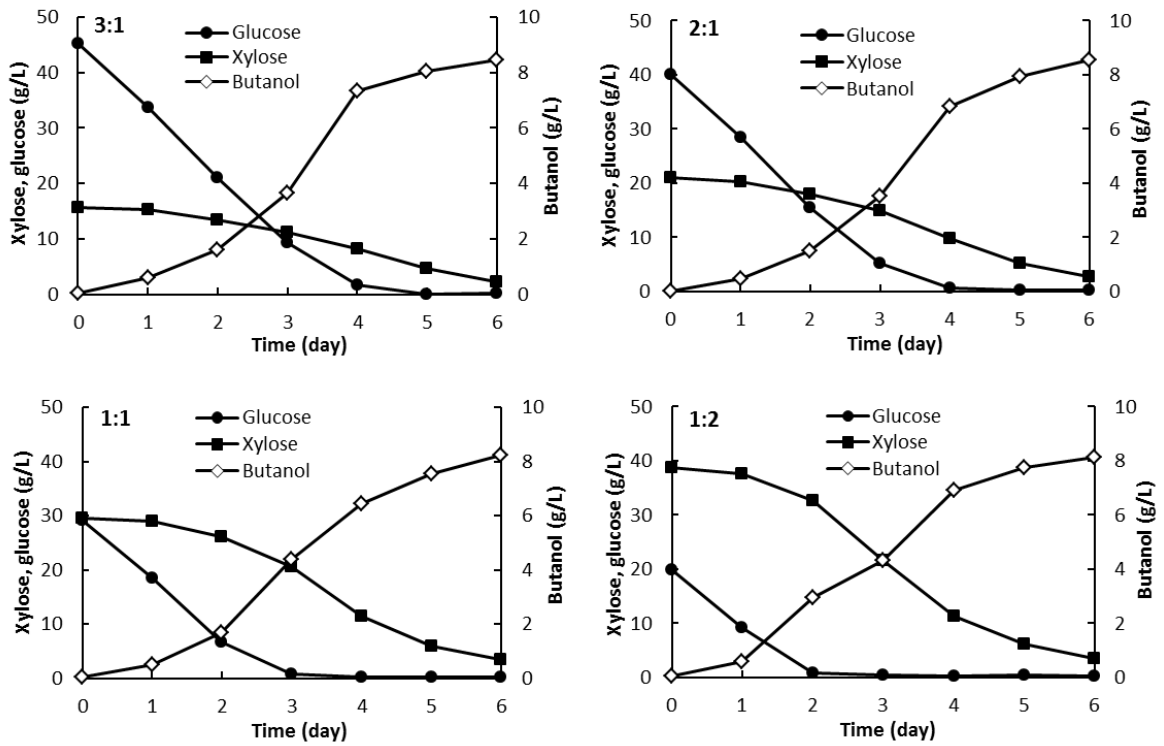

Figure 3.

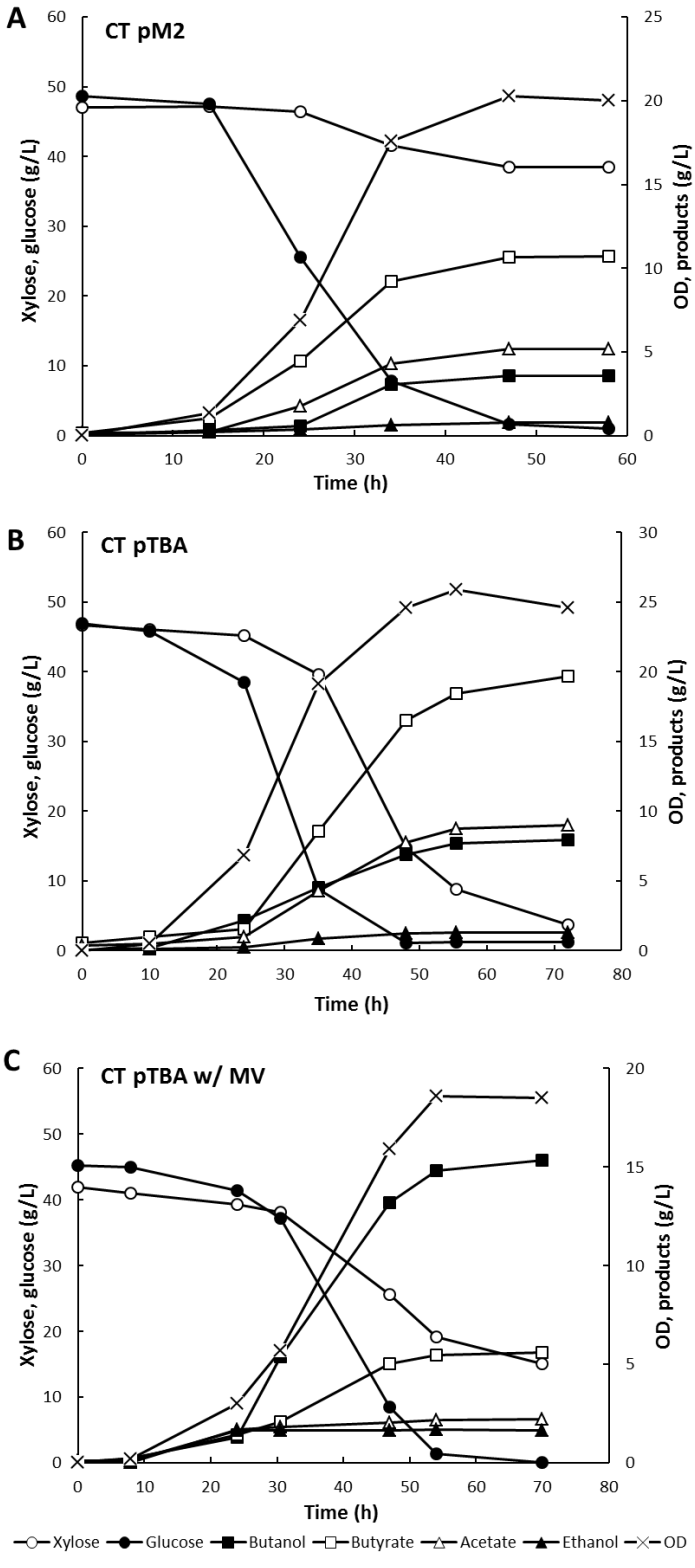

Figure 4.