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Metabolic engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid

Han Xiao [a,b], Zhilin Li [c], Yu Jiang [a,c], Yunliu Yang [a], Weihong Jiang [a], Yang Gu [a,**], Sheng Yang [a,c,*]

[a] Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China
[b] Graduate University of the Chinese Academy of Sciences, Beijing 100049, China
[c] Shanghai Research and Development Center of Industrial Biotechnology, Shanghai 201201, China

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ABSTRACT

Clostridium beijerinckii is an attractive butanol-producing microbe for its advantage in co-fermenting hexose and pentose sugars. However, this *Clostridium* strain exhibits undesired efficiency in utilizing D-xylose, one of the major building blocks contained in lignocellulosic materials. Here, we reported a useful metabolic engineering strategy to improve D-xylose consumption by *C. beijerinckii*. Gene cbei2385, encoding a putative D-xylose repressor XylR, was first disrupted in the *C. beijerinckii* NCIMB 8052, resulting in a significant increase in D-xylose consumption. A D-xylose proton-symporter (encoded by gene cbei0109) was identified and then overexpressed to further optimize D-xylose utilization, yielding an engineered strain 8052xylR-xylT$_{ptb}$ (*xylR* inactivation plus *xylT* overexpression driven by *ptb* promoter). We investigated the strain 8052xylR-xylT$_{ptb}$ in fermenting xylose mother liquid, an abundant by-product from industrial-scale xylose preparation from corncob and rich in D-xylose, finally achieving a 35% higher *A*cetone, *B*utanol and *E*thanol (ABE) solvent titer (16.91 g/L) and a 38% higher yield (0.29 g/g) over those of the wild-type strain. The strategy used in this study enables *C. beijerinckii* more suitable for butanol production from lignocellulosic materials.

1. Introduction

*A*cetone, *B*utanol and *E*thanol (ABE) fermentation is a traditional and recently reviving industrial-scale biological process (Awang et al., 1988; Durre, 1998; Gu et al., 2011). Of the three main products, butanol is not only an important bulk chemical but also an excellent transportation fuel (Durre, 2007). Butanol production through biological processes has attracted increasing attention during the past few years (Jiang et al., 2009; Yu et al., 2011). Considering the importance of using lignocellulosic resources, including forest and agricultural residues, to replace cereal substrates in production of biofuels and biochemicals (Fischer et al., 2008; Himmel and Bayer, 2009; Nicolaou et al., 2010; Somerville et al., 2010), solventogenic clostridia, once dominant in maize- or molasses-based ABE fermentation (Jones and Woods, 1986), is also required to meet the fermentation process using lignocellulosic sugars as the materials.

Among solventogenic clostridia, *Clostridium beijerinckii*, an anaerobic gram-positive *Clostridium* species, is capable of utilizing hexose and pentose sugars (e.g. D-glucose, D-xylose and L-arabinose). More importantly, no "glucose repression" effect was found when *C. beijerinckii* fermented pentose sugars in the presence of D-glucose (Ezeji et al., 2007), which confers an attractive advantage to *C. beijerinckii* over *C. acetobutylicum* (Ounine et al., 1985), another main species of solventogenic clostridia (Durre, 2008). Thus, *C. beijerinckii* could be a promising *Clostridium* strain to be improved for butanol production from lignocellulosic sugars. However, the ability of *C. beijerinckii* in using D-xylose, one of the main building blocks of lignocellulosic biomass from different sources (Aristidou and Penttila, 2000), was still undesired (Ezeji et al., 2007; Liu et al., 2010), thereby suggesting a further improvement for this *Clostridium* strain.

According to the genome sequencing result of *C. beijerinckii* NCIMB 8052 (http://www.ncbi.nlm.nih.gov; Completed: 29 June 2007), a big gene cluster (encompassing cbei2377-cbei2389) contains most of D-xylose pathway genes as well as a putative XylR-encoding gene cbei2385 (Gu et al., 2010). In some gram-positive organisms, XylR was known to specifically repress D-xylose pathway genes, and moreover, such a repression could be relieved through D-xylose induction (Gartner et al., 1992; Scheler et al., 1991). Inactivation of *xylR* enabled the expression of D-xylose pathway genes (Gartner et al., 1992; Heo et al., 2008), thereby indicating that such a *xylR*-like gene found in *C. beijerinckii* is likely to be targeted for improving D-xylose pathway. In addition, D-xylose uptake is often considered as a rate-limiting step for D-xylose metabolism in microorganisms (Gardonyi et al., 2003; Ren et al., 2009; Saloheimo et al., 2007). Due to lack of a highly efficient D-xylose transport system, many bacteria, including solventogenic clostridia, was limited in D-xylose utilization (Schmiedel and Hillen, 1996; Walmsley et al., 1998; Xiao et al., 2011). We speculated that such a bottleneck also exists in *C. beijerinckii*,

* Corresponding author at: Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China. Fax: +86 21 54924015.
** Corresponding author. Fax: +86 21 54924015.
E-mail addresses: ygu02@sibs.ac.cn (Y. Gu), syang@sibs.ac.cn (S. Yang).

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and thus, genes responsible for D-xylose uptake could be chosen here as another target for improving D-xylose metabolism.

In this work, we focused on genetic modification of *C. beijerinckii* to improve its D-xylose utilization. First, the *xylR* gene was disrupted by TargeTron technology (Shao et al., 2007), resulting in a greatly improved D-xylose consumption. Next, a *C. beijerinckii* D-xylose transporter XylT was identified and overexpressed in the *xylR*-disrupted mutant strain to further optimize D-xylose metabolism. The final recombinant strain, obtained by this combined engineering strategies, was compared to the wild-type strain to evaluate its advantage in butanol production from xylose mother liquid, an abundant by-product in the industrial-scale xylose preparation from corncob (Cheng et al., 2011). The results demonstrate that such an engineering strategy is valuable for improving *C. beijerinckii* in ABE solvent production from lignocellulosic materials.

2. Materials and methods

2.1. Strains, plasmids, media and cultivation conditions

The bacterial strains and plasmids used in the study are listed in Table 1. *Escherichia coli* cells were grown at 37 °C in Luria–Bertani medium or on Luria–Bertani agar [Luria–Bertani plus 1.5% (wt/vol) Difco agar]. *C. beijerinckii* cells were grown anaerobically (Thermo Forma Inc., Waltham, MA) at 37 °C in liquid *Clostridium* growth medium (CGM) or on solid CGM medium [plus 2% (wt/vol) Difco agar] (Wiesenborn et al., 1988). The M9 medium was also used for *E. coli* growth.

The XHP2 medium, which was modified from P2 medium (Baer et al., 1987), was used for *C. beijerinckii* growth. The difference between XHP2 and P2 medium was the solution II. Components of solution II in XHP2 were 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 2.14 g/L NH_4Cl and 7.85 g/L CH_3COOK. 100 μg/mL ampicillin and 10 μg/mL erythromycin were used for *E. coli* and *C. beijerinckii*, respectively, when they are needed. *C. beijerinckii* strain was maintained in 20% (vol/vol) glycerol and stored at −20 °C. The xylose mother liquid used in this study was provided from Shandong Longlive Biotechnology Co. Ltd. (High-technology Development Zone of Yucheng, Shandong Province, China). The xylose mother liquid medium for ABE fermentation was prepared according to the ingredients of XHP2 medium except for the carbon sources.

SP2 medium, obtained by adding 0.1 g/L L-cysteine and 6 g/L yeast extract into XHP2 and using glycerin (30 g/L) as the carbon source, was adopted for culturing cells for RT-PCR analysis.

2.2. Construction of plasmids pUC118-xylT, pWJ1-xylR, pWJ1-xylT, pIMP1-$xylR_{xylR}$, pIMP1-$xylT_{adc}$, pIMP1-$xylT_{ptb}$, pIMP1-$xylT_{thl}$, pIMP1-$lacZ_{adc}$, pIMP1-$lacZ_{ptb}$, and pIMP1-$lacZ_{thl}$

Construction of plasmid pUC118-xylT was performed as following: the full-length coding region of gene cbei0109 was

Table 1
Bacterial strains and plasmids.

Strains or plasmids	Relevant characteristics[a]	Reference or source[b]
Strains		
C. acetobutylicum ATCC 824	Wild-type	ATCC
C. beijerinckii NCIMB 8052	Wild-type	NCIMB
8052xylR	xylR::intron/pWJ1-xylR	This study
8052xylR-P	8052xylR/pIMP1-P_{ptb}	This study
8052xylR-X	8052xylR/pIMP1-$xylR_{xylR}$	This study
8052xylT	xylT::intron/pWJ1-xylT	This study
8052WT-P	8052WT/pIMP1-P_{ptb}	This study
8052WT-$xylT_{thl}$	8052WT/pIMP1-$xylT_{thl}$	This study
8052xylR-$xylT_{adc}$	8052xylR/pIMP1-$xylT_{adc}$	This study
8052xylR-$xylT_{ptb}$	8052xylR/pIMP1-$xylT_{ptb}$	This study
8052xylR-$xylT_{thl}$	8052xylR/pIMP1-$xylT_{thl}$	This study
8052xylR-$lacZ_{adc}$	8052xylR/pIMP1-$lacZ_{adc}$	This study
8052xylR-$lacZ_{ptb}$	8052xylR/pIMP1-$lacZ_{ptb}$	This study
8052xylR-$lacZ_{thl}$	8052xylR/pIMP1-$lacZ_{thl}$	This study
E. coli DH5α	General cloning host strain	Takara
E. coli K-12	Wild type	Takara
E. coli K12-P	*E. coli* K12 bearing empty plasmid pUC118	This study
E. coli K12xylE	*E. coli* K12 with *xylE* inactivated	CGSC
E. coli K12xylE-P	*E. coli* K12xylE bearing empty plasmid pUC118	This study
E. coli K12xylE-xylT	*E. coli* K12xylE bearing *C. beijerinckii* gene cbei0109	This study
Plasmids		
pUC118	Expression vector in *E.coli*	Novagen
pUC118-xylT	Derived from pUC118, with *xylT* gene (cbei0109) expressing cassette added	This study
pWJ1-xylR	Derived from pWJ1 for intron insertion in *xylR* at 787/788 nt	This study
pWJ1-xylT	Derived from pWJ1 for intron insertion in *xylT* at 852/853 nt	This study
pIMP1-$xylR_{xylR}$	Derived from pIMP1, with xylR gene (cbei2385) and its promoter expression cassette added	This study
pIMP1-P_{ptb}	ColE1 ORI, Amp^r, pIM13 ORI, MLS^r, *ptb* (cac3076) promoter region of *C. acetobutylicum* ATCC 824	Offered by Prof. Papoutsakis E.T. (Xiao et al., 2011)
pIMP1-P_{thl}	ColE1 ORI, Amp^r, pIM13 ORI, MLS^r, *thl* (cac2873) promoter region of *C. acetobutylicum* ATCC 824	
pIMP1-P_{adc}	Derived from pIMP1-P_{thl}, with *adc* (cap0165) promoter instead of thl promoter	This study
pIMP1-$xylT_{adc}$	Derived from pIMP1-P_{adc}, with *xylT* gene (cbei0109) expressing cassette added	This study
pIMP1-$xylT_{ptb}$	Derived from pIMP1-P_{ptb}, with *xylT* gene (cbei0109) expressing cassette added	This study
pIMP1-$xylT_{thl}$	Derived from pIMP1-P_{thl}, with *xylT* gene (cbei0109) expressing cassette added	This study
placZFT	Amp^r, *lacZ* gene from *Thermoanaerobacterium thermosulfurogenes* EM1	Offered by Prof. Papoutsakis E.T.
pIMP1-$lacZ_{adc}$	Derived from pIMP1-P_{adc}, with *lacZ* gene expressing cassette added	This study
pIMP1-$lacZ_{ptb}$	Derived from pIMP1-P_{ptb}, with *lacZ* gene expressing cassette added	This study
pIMP1-$lacZ_{thl}$	Derived from pIMP1-P_{thl}, with *lacZ* gene expressing cassette added	This study

[a] XylR, transcriptional regulator of D-xylose metabolism; XylT, D-xylose transporter; LtrA, LtrA protein, required for trans-splicing; ColE1 ORI, ColE1 origin of replication; Amp^r, ampicillin resistance; pIM13 ORI, gram-positive origin of replication; MLS^r, macrolide–lincosamide–streptogramin resistance; Ptb, phosphotransbutyrylase; Thl, thiolase; Adc, acetoacetate decarboxylase.

[b] ATCC, American Type Culture Collection, America; CGSC, The Coli Genetic Stock Center, America; NCIMB, The National Collection of Industrial, Marine and Food Bacteria, Scotland.

Table S1
Primers used in this study.

Primer name	Sequence (5'-3')	Description
118-xylT-up	CGGGGTACCCATGAAAATAAAAATTAGTAAC	Forward primer for PCR of full-length coding region of gene cbei0109
118-xylT-dn	ACGCGTCGACCTAAGCCTTAGTTGTAGCATC	Reverse primer for PCR of full-length coding region of gene cbei0109
xylR787|788s–IBS	AAAACTCGAGATAATTATCCTTACATACCCATCTAGTGCGCCCAGATAGGGTG	*xylR* Targetron primer[a]
xylR787|788s–EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATCTATGTAACTTACCTTTCTTTGT	*xylR* Targetron primer[a]
xylR787|788s–EBS2	TGAACGCAAGTTTCTAATTTCGGTTGTATGTCGATAGAGGAAAGTGTCT	*xylR* Targetron primer[a]
xylR_569–588	ATTTATCTGCTTACTACGAG	Forward primer inside *xylR* from 569 to 588 base
xylR_918–937	AATTCGATAGACCTAAAGAC	Reverse primer inside *xylR* from 918 to 937 base
xylR-up	AAAACTGCAGATATATTAAAGATAGAAATCAATCATCTTAG	*xylR* Promoter forward primer
xylR-dn	CGGGGTACCTTATTTAATTTCTAAGAATTCTTCTATAGGC	*xylR* Reverse primer
xylT852|853s–IBS	AAAACTCGAGATAATTATCCTTAGCTTTCCTCGCTGTGCGCCCAGATAGGGTG	*xylT* Targetron primer[a]
xylT852|853s–EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTCGCTCATAACTTACCTTTCTTTGT	*xylT* Targetron primer[a]
xylT852|853s–EBS2	TGAACGCAAGTTTCTAATTTCGATTAAAGCTCGATAGAGGAAAGTGTCT	*xylT* Targetron primer[a]
xylT_579–596	AGATGGACGAGCAGATGA	Forward primer inside *xylT* from 579 to 596 base
xylT_1006–1023	GATTGAGGCAACGAAAGA	Reverse primer inside *xylT* from 1006 to 1023 base
adc-up	AACTGCAGTAAAAACACCTCCACATAAG	*adc* Promoter forward primer
adc-dn	ACGCGTCGACAAAAGTCACCTTCCTAAATTTAA	*adc* Promoter reverse primer
xylT-up	ACGCGTCGACATGAAAATAAAAATTAGTAACTCTG	*xylT* Forward primer
xylT-dn	CGGGGTACCCTAAGCCTTAGTTGTAGCATCTTGAGTG	*xylT* Reverse primer
lacZ-up	CGCGGATCCATGAGAAAGATTATTCC	*lacZ* Forward primer
lacZ-dn	GGACCCGGGAGATGAAATTCTCTTTC	*lacZ* Reverse primer
dpIMP1-fw	GCAAGAGGCAAATGAAATAG	Forward primer on pIMP1
dpIMP1-rev	TGCTGCAAGGCGATTAAGTTGG	Reverse primer on pIMP1
dxylR-dn	TCGGTGTTAGTGCGACTCC	Reverse primer inside *xylR*
dpIMP1-P_{adc}-up	TAGGACTAGAGGCGATTT	Forward primer inside adc promoter on pIMP1-P_{adc}
dpIMP1-P_{ptb}-up	TAAATGAGCACGTTAATC	Forward primer inside ptb promoter on pIMP1-P_{ptb}
dpIMP1-P_{thl}-up	TTAGGGATAAACTATGGAAC	Forward primer inside thl promoter on pIMP1-P_{thl}
dxylT-dn	CAGCCGATGTAAGAATTAGC	Reverse primer inside *xylT*
dlacZ-dn	CTTCCAGTCATAAACCTC	Reverse primer inside *lacZ*
rxylF-up	ACTAATCCATATTGGCTTGA	Forward RT-PCR primer for *xylF*
rxylF-dn	TATGCCACTCACCTTTGC	Reverse RT-PCR primer for *xylF*
rxylAI-up	GGTGGACGTGAAGGATAT	Forward RT-PCR primer for *xylAI*
rxylAI-dn	TATTGGTGCTTTGTTGGT	Reverse RT-PCR primer for *xylAI*
rxylAII-up	TCTTAGCTTGTAATGCGCCT	Forward RT-PCR primer for *xylAII*
rxylAII-dn	ATGCTAGCTATAACGTCTGG	Reverse RT-PCR primer for *xylAII*
rxylB-up	GCGGCAACTGATACACCT	Forward RT-PCR primer for *xylB*
rxylB-dn	CTCGCTTTCTTCAACTTCTTTA	Reverse RT-PCR primer for *xylB*
rtal-up	ATAGAAGCAGCAAAAGCAGGC	Forward RT-PCR primer for *tal*
rtal-dn	GCTGCTGCCCAGTCTGCTTTA	Reverse RT-PCR primer for *tal*
rxylT-up	AGATGGACGAGCAGATGA	Forward RT and real-time PCR primer for *xylT*
rxylT-dn	AACCAAAGCCTATGACTA	Reverse RT and real-time PCR primer for *xylT*
r16S–up	CGCACAAGCAGCGGAGCAT	Forward RT and real-time PCR primer for cbeir0001
r16S–dn	AACCCAACATCTCACGACACGA	Reverse RT and real-time PCR primer for cbeir0001

[a] Designed by on-line tools (www.clostron.com).

obtained by PCR using 118-xylT-up/118-xylT-dn as primers (Table S1). The PCR fragment was digested with *Acc65*I and *Sal*I, and then cloned into the pUC118 digested with the same restriction enzymes, yielding the plasmid pUC118-xylT.

The 350 bp TargeTron fragment for *xylR* gene was amplified by using primers xylR787|788s–IBS, xylR787|788s–EBS1d and xylR787|788s–EBS2 according to the protocol of the TargeTron™ Gene Knockout System Kit (Sigma-Aldrich, St Louis, MO, USA). The plasmid pWJ1-xylR was obtained by inserting this 350 bp fragment into the *Xho*I and *BsrG*I sites of the plasmid pWJ1 (Table 1). All primers involved in constructing the pWJ1-xylR plasmid are listed in Table S1. The primers IBS, EBS1d and EBS2 were designed using the Clostron tool (http://www.clostron.com). The plasmid pWJ1-xylT was constructed in the same way using the primers xylT852|853s–IBS, xylT852|853s–EBS1d and xylT852|853s–EBS2 (Table S1).

The putative *xylR* gene (cbei2385) together with its promoter region was amplified via PCR using the primers pairs of xylR-up/xylR-dn (Table S1). The *xylR* fragment was then digested with *Pst*I and *Acc65*I, and cloned into the plasmid pIMP1-P_{thl} digested with the same restriction enzymes, yielding the plasmid pIMP1-$xylR_{xylR.}$

The promoter region of *adc* gene (cap0165, encoding acetoacetate decarboxylase) of *C. acetobutylicum* was obtained by PCR using the primers adc-up/adc-dn (Table S1). The PCR fragment containing was digested with *Pst*I and *Sal*I, and then inserted into the plasmid pIMP1-P_{thl}, which was digested with the same restriction enzymes, yielding the plasmid pIMP1-P_{adc}. The *xylT* gene (cbei0109) was amplified via PCR using the primer pairs of xylT-up/xylT-dn (Table S1), then digested with *Sal*I and *Acc65*I and cloned into the plasmid pIMP1-P_{adc}, pIMP1-P_{ptb} and pIMP1-P_{thl}, which were digested with the same restriction enzymes, yielding the plasmid pIMP1-$xylT_{adc}$, pIMP1-$xylT_{ptb}$ and pIMP1-$xylT_{thl}$, respectively. The *lacZ* gene was obtained by PCR using the plasmid placZFT as the template and lacZ-up/lacZ-dn as the primers (Table S1). The PCR fragment of *lacZ* gene was digested with *BamH*I and *Sma*I, and then inserted into the plasmid

pIMP1-P_{adc} pIMP1-P_{ptb} and pIMP1-P_{thl}, which were digested with the same restriction enzymes, yielding the plasmid pIMP1-$lacZ_{adc}$, pIMP1-$lacZ_{ptb}$ and pIMP1-$lacZ_{thl}$, respectively.

2.3. Electroporation of C. beijerinckii and identification of mutants

For *xylR* inactivation, the plasmid was electroporated into cells (Mermelstein et al., 1992); cells were plated on CGM agar containing 10 μg/mL erythromycin and incubated anaerobically at 37 °C for 48 h. Identification of the positive transformants containing the insert was performed by colony PCR using the primer xylR_569-588/xylR_918-937 (Table S1). The PCR fragment was sequenced to confirm the insertion of intron. To eliminate erythromycin resistance for the next-step plasmid electroporation, the *xylR* gene-disrupted strain, harboring the plasmid pWJ1-xylR, was successively transferred (once every 12 h) in liquid CGM medium without antibiotics at 37 °C for 1–2 days. Cells were then plated on CGM agar and incubated to obtain individual colonies. The colonies losing plasmids were confirmed by colony PCR using the primer xylR_569-588/xylR_918-937 (Table S1). The resulting mutant strain was named as 8052xylR. As to *xylT* inactivation, same method was used except that colony PCR for identification of transformants containing intron inserts used primer xylT_579-596/xylT_1006-1023 (Table S1).

For gene overexpression in *C. beijerinckii*, the plasmids were introduced into cells by electroporation and then identified via colony PCR. The plasmids and the primers used for colony PCR were as following: pIMP1-P_{ptb}, dpIMP1-P_{ptb}-up/dpIMP1-rev; pIMP1-$xylT_{thl}$, dpIMP1-P_{thl}-up/dxylT-dn; pIMP1-P_{ptb}, dpIMP1-P_{ptb}-up/dpIMP1-rev; pIMP1-$xylR_{xylR}$, dpIMP1-fw/dxylR-dn; pIMP1-$xylT_{adc}$, dpIMP1-P_{adc}-up/dxylT-dn; pIMP1-$xylT_{ptb}$, dpIMP1-P_{ptb}-up/dxylT-dn; pIMP1-$xylT_{thl}$, dpIMP1-P_{thl}-up/dxylT-dn; pIMP1-$lacZ_{adc}$, dpIMP1-P_{adc}-up/dlacZ-dn; pIMP1-$lacZ_{ptb}$, dpIMP1-P_{ptb}-up/dlacZ-dn; pIMP1-$lacZ_{thl}$, dpIMP1-P_{thl}-up/dlacZ-dn.

2.4. RNA preparation, RT-PCR and q-PCR analysis

Samples for RT-PCR analysis were collected from cells grown in SP2 medium. Samples for real-time PCR analysis were collected from XHP2 medium using 60 g/L D-xylose as the carbon source. RNA preparation, generation of cDNA, RT-PCR and real-time PCR analysis were performed as described previously (Pfaffl, 2001; Ren et al., 2010; Xiao et al., 2011). The gene cbeir0001 (encoding 16S ribosomal RNA) was used as an internal control for RT-PCR and real-time PCR.

2.5. β-galactosidase activity assay

C. beijerinckii 8052xylR strain and its derivative strains were grown in 100 mL of XHP2 medium using 60 g/L D-xylose as the carbon source. Pellets of 5 mL of cells were collected by centrifugation at 4 °C and then frozen at −80 °C. Crude extracts were harvested and processed as described previously (Tummala et al., 1999). The β-galactosidase assay was performed as described by Miller, except that 60 °C was used as the assay temperature (Miller, 1972).

2.6. Complementation analysis in E. coli

The plasmid pUC118-xylT was transformed into *E. coli* K12xylE, the *xylE*-inactivated mutant strain, for the complementation analysis. The empty vector was expressed in both the *E. coli* K12xylE and wild type K-12 strain, yielding the *E. coli* K12xylE-P and K12-P respectively. Strain K12-P and K12xylE-P were used as positive and negative control. Complementation experiments were carried out in M9 medium containing 5 g/L D-xylose as described previously (Gu et al., 2010).

2.7. Fermentations

Fermentations using XHP2 medium were performed as following: 100 μl of frozen stock was inoculated into 5 mL CGM liquid medium and incubated anaerobically at 37 °C for 24 h. When the optical density (OD_{600}) of cells reached 0.5–1.0, 5 mL of the grown cells were inoculated into 95 mL of XHP2 medium containing 60 g/L of D-xylose as the carbon source. When optical density (OD_{600}) of the cells reached 0.8–1.0, about 5% (vol/vol) of the inoculum was transferred into 95 mL of XHP2 medium or xylose mother liquid medium (36.08 g/L D-xylose, 7.18 g/L D-glucose and 11.33 g/L L-arabinose, as well as all components contained in XHP2 except of carbon sources) for fermentation.

The yield was defined as total grams of ABE solvents produced per total grams of initial carbon sources (sugars and acetate).

2.8. Analytical methods

Cell density (OD_{600}) was determined at A_{600} using a DU730 spectrophotometer (Beckman Colter). Solvents (acetone, butanol and ethanol) were determined by gas chromatography (7890 A, Agilent, Wilmington, DE, USA). Concentrations of D-glucose, D-xylose and L-arabinose were determined by using a high-pressure liquid chromatography (HPLC) system (1200 series, Agilent). Conditions of gas and liquid chromatographs were the same as described previously (Ren et al., 2010).

3. Results

3.1. Disruption of the gene cbei2385 improves D-xylose utilization by C. beijerinckii

Although no "glucose repression" was observed in *C. beijerinckii* when fermenting hexose and pentose sugars, D-xylose utilization was undesired for this strain (Ezeji et al., 2007; Qureshi et al., 2008). While grown in XHP2 medium using D-xylose as the sole carbon source (30 g/L), *C. beijerinckii* NCIMB 8052 could only consume 20.20 g/L D-xylose after 72 h (Fig. S1), indicative of inherent D-xylose metabolism bottlenecks in this strain.

According to the genome annotation (http://www.ncbi.nlm.nih.Gov; Completed: 29 June 2007), gene cbei2385, encoding a putative XylR repressor, is clustered with D-xylose pathway genes on the chromosome. The candidate regulatory sites of XylR has also been found in the upstream regions of most of these D-xylose pathway genes (Gu et al., 2010). To investigate whether XylR regulator is involved in regulating D-xylose metabolism in *C. beijerinckii*, gene cbei2385 was inactivated by using TargeTron system (Shao et al., 2007), yielding a mutant strain 8052xylR. The intron insertion of this gene was confirmed by sequencing (Fig. S2).

The inactivation of *xylR* gene caused significant up-regulation in expression levels of most of the D-xylose pathway genes, and thereby, contributed to D-xylose utilization. As shown in Fig. 1, after *xylR* inactivation, gene cbei0109 (encoding sugar transporter), cbei2383 (encoding type I D-xylose isomerase), cbei4681 (encoding type II D-xylose isomerase) and cbei2384 (encoding xylulokinase) all exhibited enhancement in the transcriptional level. However, no obvious expressional variations were found in gene cbei2377 (encoding D-xylose transporter) and cbei2386 (encoding transaldolase) (Fig. 1).

The ability of the mutant strain (8052xylR) and the wild-type strain (8052WT) in D-xylose utilization were also investigated. The results showed that the 8052xylR had obvious advantages in

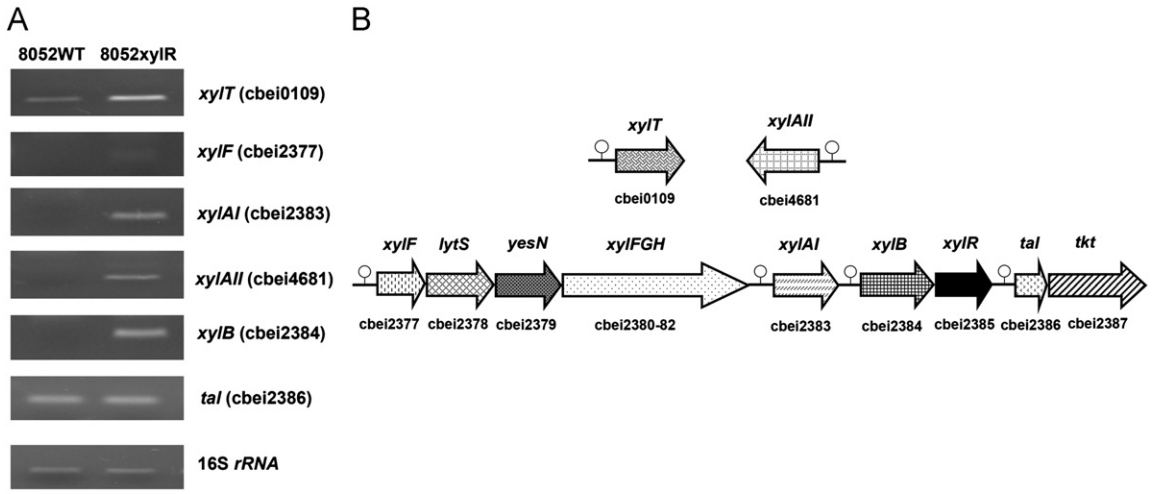

Fig. 1. (A) Comparison of transcriptional level of gene *xylT* (cbei0109), *xylF* (cbei2377), *xylAI* (cbei2383), *xylAII* (cbei4681), *xylB* (cbei2384) and *tal* (cbei2386) gene between the strains 8052WT and 8052xylR. Samples were collected from SP2 medium at OD_{600}=0.5. 16S *rRNA* gene (cbeir0001) was used as the loading control. (B) The chromosomal cluster and putative regulon containing D-xylose pathway genes in *C. beijerinckii*. Candidate regulatory sites of XylR are shown by circles (Gu et al., 2010).

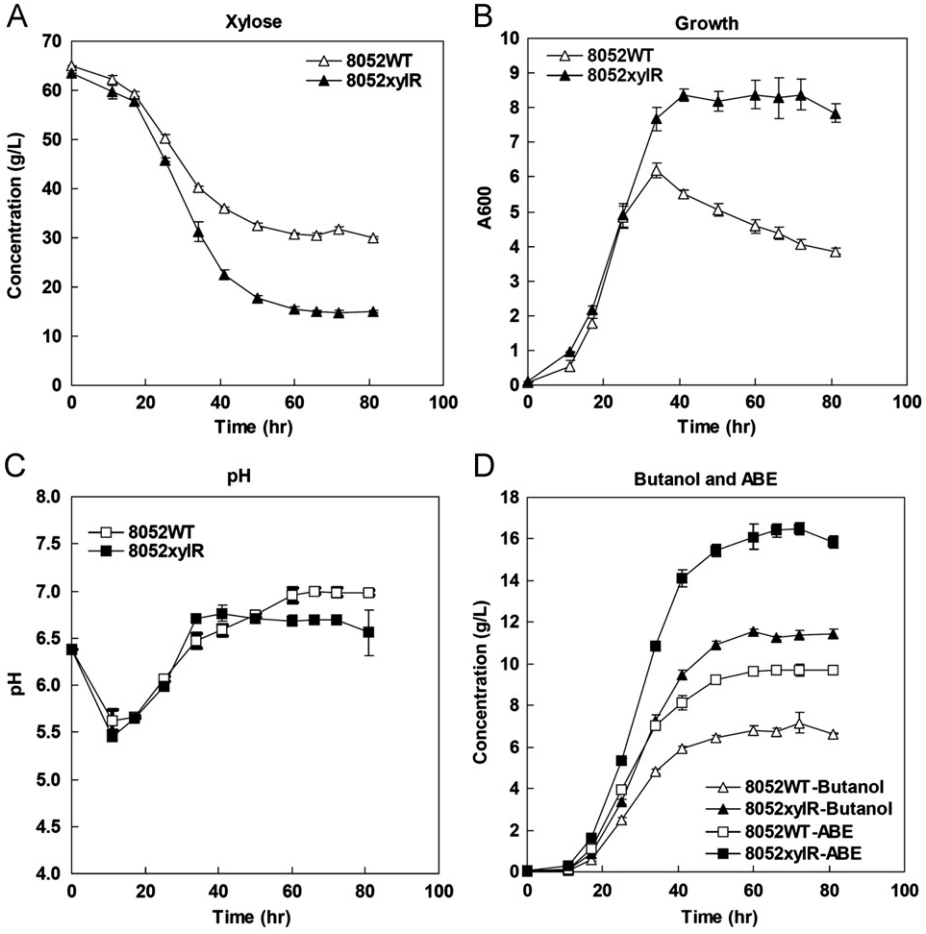

Fig. 2. Growth and metabolite profiles of the 8052WT and 8052xylR strain in batch fermentations (XHP2 medium containing 60 g/L D-xylose as the carbon source). (A) Sugars consumption; (B) Growth; (C) pH values; (D) Butanol and ABE concentrations. Fermentations were performed in triplicate.

growth and D-xylose consumption over the 8052WT (Fig. 2A and B), while the pH variation curve of these two strains were similar (Fig. 2C). At the end of fermentation, the residual D-xylose concentration for the 8052xylR was 15.08 g/L, namely 76% of the total amount of D-xylose was consumed, whereas that attained by the 8052WT was only 54% (Fig. 2A). As a result, the 8052xylR was able to produce 11.40 g/L butanol and 16.50 g/L total solvents within 72 h, a significant increase compared to 7.17 g/L butanol and 9.69 g/L total solvents observed for the 8052WT (Fig. 2D).

The complementation experiment was also carried out to examine whether such an improvement of D-xylose consumption

Table 2
Complementation of the 8052xylR strain with gene cbei2385.

Strains[a]	D-Xylose consumption (g/L)	Butanol (g/L)	ABE (g/L)
8052WT	34.26 ± 0.64	7.51 ± 0.30	9.01 ± 0.48
8052xylR	44.40 ± 3.70	9.68 ± 1.25	11.35 ± 0.78
8052xylR-P	43.90 ± 1.62	9.79 ± 0.43	12.47 ± 0.83
8052xylR-X	35.17 ± 1.00	7.17 ± 0.74	9.53 ± 1.85

[a] 8052WT: *C. beijerinckii* NCIMB 8052 wild-type strain; 8052xylR: *cbei2385*-disrupted strain; 8052xylR-P: *cbei2385*-disrupted strain harboring plasmid pIMP1-P_{ptb}; 8052xylR-X: *cbei2385*-disrupted strain harboring plasmid pIMP1-$xylR_{xylR}$. Batch fermentations were performed in XHP2 medium containing 60 g/L D-xylose as the carbon source. Samples were taken at 96 h. Fermentations were performed in triplicate.

in the 8052xylR was really caused by inactivation of gene cbei2385. As shown in Table 2, when gene cbei2385 was re-introduced into the 8052xylR, the resulting 8052xylR-X strain showed a comparative level to that of the wild-type strain (8052WT) in D-xylose consumption, butanol and solvent production. No significant difference was found between the 8052xylR and 8052xylR-P, indicative of no plasmid effect here (Table 2).

3.2. The gene cbei0109 encodes a D-xylose transporter in C. beijerinckii NCIMB 8052

As D-xylose uptake has been regarded as a rate-limiting step in D-xylose pathway in many microbes (Jojima et al., 2010), we thus tried to strengthen this step in *C. beijerinckii* 8052xylR to further improve D-xylose consumption. According to the genome annotation, a total of 14 genes were predicted as sugar transporters in *C. beijerinckii* NCIMB 8052, and gene cbei2380 was the only one annotated as a D-xylose transporter. However, gene cbei0109 was deemed to be a D-xylose transporter here because it shared the highest amino acid sequence homology (37%) with a previously characterized D-xylose transporter gene (cac1345) in *C. acetobutylicum* ATCC 824 (Gu et al., 2010) (Table S2). Moreover, the result of functional complementary also showed that gene cbei0109 could restore the ability of *E. coli* Δ*xylE* mutant to utilize D-xylose, thus indicating the D-xylose transporter role gene cbei0109 plays in *C. beijerinckii* (Fig S3). To further verify the contribution of this inferred transporter gene on D-xylose utilization, cbei0109 was: (*i*) inactivated by inserting an intron (using the TargeTron system), yielding the strain 8052xylT; (*ii*) overexpressed under the control of the *thl* promoter using plasmid (Tomas et al., 2003), yielding the strain 8052-$xylT_{thl}$. These two recombinant strains were compared to the wild-type strain 8052WT in fermenting D-xylose (60 g/L). After 96 h, the 8052WT, 8052xylT and 8052-$xylT_{thl}$ consumed 32.17, 17.76 and 42.80 g/L D-xylose, respectively. All these results demonstrates the important role gene cbei0109 plays in D-xylose transport in *C. beijerinckii*, and we thus named gene cbei0109 as *xylT*.

3.3. XylT overexpression in the strain 8052xylR further enhances D-xylose consumption

Three promoters, which have been identified in *C. acetobutylicum* (Girbal et al., 2003; Tummala et al., 1999), namely *adc*, *ptb* and *thl*, were chosen here to drive *xylT* expression in the 8052xylR, yielding three recombinant strains 8052xylR-$xylT_{adc}$, 8052xylR-$xylT_{ptb}$ and 8052xylR-$xylT_{thl}$, respectively. All the promoters showed driving strengths in *C. beijerinckii* according to the β-galactosidase (β-Gal) activity assays (Fig. 3). Interestingly, only the 8052xylR-$xylT_{ptb}$ exhibited significantly enhanced D-xylose consumption (Fig. 4), cell growth and solvent production (Table S3) in comparison with the strain 8052xylR when fermenting

Table S2
Amino acid identity Matrix of 14 putative sugar transporters in *C. beijerinckii* NCIMB 8052 and XylT (cac1345) in *C. acetobutylicum* ATCC 824. All data were obtained through the software Bioedit.

	cbei0109	cbei0232	cbei0770	cbei0771	cbei1835	cbei2377	cbei2380	cbei3764	cbei4125	cbei4443	cbei4448	cbei4461	cbei4545	cbei4588	XylT(cac1345)
cbei0109	–	0.07	0.08	0.06	0.09	0.05	0.05	0.12	0.10	0.06	0.09	0.08	0.35	0.08	0.37
cbei0232	–	–	0.12	0.32	0.06	0.05	0.04	0.07	0.07	0.24	0.05	0.05	0.07	0.05	0.05
cbei0770	–	–	–	0.10	0.03	0.07	0.04	0.06	0.06	0.11	0.04	0.06	0.05	0.05	0.05
cbei0771	–	–	–	–	0.04	0.07	0.06	0.05	0.05	0.22	0.06	0.06	0.06	0.06	0.05
cbei1835	–	–	–	–	–	0.05	0.05	0.21	0.26	0.04	0.08	0.07	0.10	0.10	0.10
cbei2377	–	–	–	–	–	–	0.13	0.06	0.08	0.05	0.06	0.05	0.06	0.04	0.06
cbei2380	–	–	–	–	–	–	–	0.07	0.07	0.05	0.06	0.05	0.05	0.07	0.05
cbei3764	–	–	–	–	–	–	–	–	0.31	0.05	0.06	0.06	0.11	0.13	0.10
cbei4125	–	–	–	–	–	–	–	–	–	0.04	0.08	0.06	0.10	0.11	0.11
cbei4443	–	–	–	–	–	–	–	–	–	–	0.05	0.03	0.07	0.08	0.06
cbei4448	–	–	–	–	–	–	–	–	–	–	–	0.07	0.09	0.08	0.07
cbei4461	–	–	–	–	–	–	–	–	–	–	–	–	0.09	0.07	0.07
cbei4545	–	–	–	–	–	–	–	–	–	–	–	–	–	0.09	0.33
cbei4588	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.08
XylT(cac1345)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

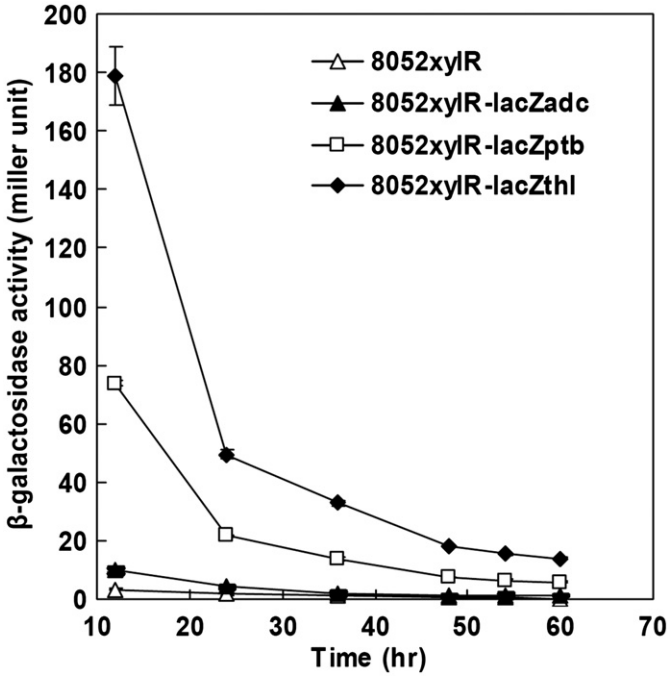

Fig. 3. β-galactosidase (β-Gal) activities resulting from *adc*, *ptb*, *thl* promoter in strain 8052xylR. These three promoters were fused with *lacZ* and subjected to β-Gal assays. Samples were taken from XHP2 medium containing 60 g/L D-xylose as the carbon source. Detections of β-Gal activities were performed in triplicate.

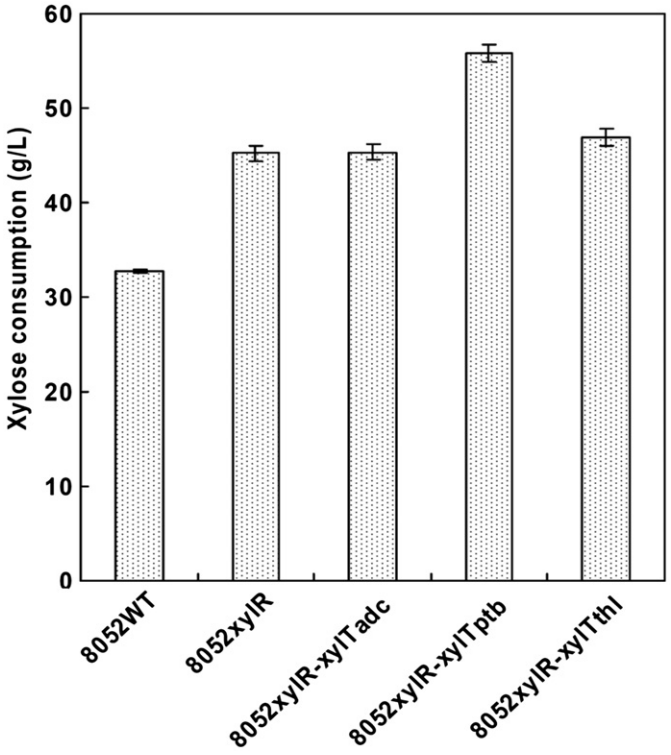

Fig. 4. Sugar consumption by the 8052WT, 8052xylR, 8052xylR-xylT$_{adc}$, 8052xylR-xylT$_{ptb}$ and 8052xylR-xylT$_{thl}$ strain. The XHP2 medium (containing 60 g/L D-xylose as the carbon source) was used here. Samples for assays were taken at 96 h. Fermentations were performed in triplicate.

D-xylose (60 g/L). The transcriptional levels of *xylT* gene of the 8052xylR and three derivative strains were then examined. The result showed that three derivative strains all had obvious increases in *xylT* transcript over the 8052xylR, except that in solventogenic phase by the 8052xylR-xylT$_{adc}$. This demonstrated an efficient plasmid expression of *xylT* gene in the 8052xylR-xylT$_{ptb}$ (Table 3).

3.4. *C. beijerinckii 8052xylR-xylT$_{ptb}$ showed significant advantage over the 8052WT in fermenting xylose mother liquid*

In order to further compare the fermentation characteristics of the 8052xylR-xylT$_{ptb}$ and 8052WT, we tested these two strains in the xylose mother liquid, containing 7.18 g/L D-glucose, 36.08 g of D-xylose and 11.33 g/L L-arabinose as the carbon sources. While no significant differences were found in pH variation and D-glucose consumption between these two strains throughout the entire fermentation course (Fig. 5A and C), the 8052xylR-xylT$_{ptb}$ displayed much better than the 8052WT in growth and D-xylose consumption (Fig. 5A and B). Interestingly, the 8052xylR-xylT$_{ptb}$ also consumed 2.98 g/L more L-arabinose than the 8052WT (Fig. 5A). Therefore, the 8052xylR-xylT$_{ptb}$ finally consumed 48.30 g/L sugars (over 88% of the total sugars) within 61 h, whereas the 8052WT only used 34.29 g/L during the same period (Fig. 5A). As a result, the 8052xylR-xylT$_{ptb}$ reached a much higher products level (10.67 g/L butanol and 16.91 g/L total solvents) compared to those (8.04 g/L butanol and 12.53 g/L total solvents) of the 8052WT (Fig. 5D). Based on the total carbon sources (54.59 g/L of mixed sugars and 4.7 g/L of acetate) in the medium, the ABE solvent yield of the 8052xylR-xylT$_{ptb}$ achieved 0.29 g/g, which was 38% higher than that (0.21 g/g) of the 8052WT. The 8052xylR was also investigated in fermenting xylose mother liquid. Compared to the 8052xylR-xylT$_{ptb}$, the 8052xylR showed lower solvent production and pentose consumption (Table S4), indicating that *xylT* overexpression did further contribute to D-xylose and L-arabinose consumption over *xylR* inactivation.

4. Discussion

In this study, we presented a strategy for metabolic engineering of D-xylose pathway in *C. beijerinckii*, an important industrial *Clostridium* strain, for ABE solvent production from lignocellulosic materials. Inactivation of the gene cbei2385, encoding a XylR-like regulator, in combination with overexpression of a newly identified *xylT* gene realized significantly enhanced D-xylose utilization by *C. beijerinckii*. To our knowledge, this is the first report focusing on overcoming D-xylose metabolism bottlenecks in *C. beijerinckii*. Given that *C. beijerinckii* NCIMB 8052 used in this study is a model strain, this strategy can be extended to many other *C. beijerinckii* strains to improve D-xylose utilization.

The putative *xylR* gene (cbei2385) was chosen here as the target because XylR has been observed in some other gram-positive bacteria to be a specific repressor of D-xylose pathway genes (Rygus and Hillen, 1992; Stephens et al., 2007). However, just as described in *Bacillus subtilis*, *Staphylococcus xylosus* and *Bacillus megaterium* (Dahl and Hillen, 1995; Kraus et al., 1994; Rygus and Hillen, 1992; Sizemore et al., 1992), such a repression could be released by D-xylose, acting as an inducer at very low concentrations. Therefore, under a relative high D-xylose concentration in the fermentation broth, (such as 60 g/L in this study, a sugar concentration often used in ABE fermentation), whether such a repression of *xylR* on D-xylose pathway genes could be thoroughly released is unknown. The result here that *xylR* inactivation could confer an improved D-xylose metabolism to *C. beijerinckii* demonstrated that such a XylR-DNA binding was not thoroughly released only by D-xylose induction, thereby highlighting the importance of *xylR*-like gene (cbei2385) in metabolic engineering of D-xylose pathway of *C. beijerinckii*.

Table S3
Fermentation parameters of strain 8052WT, 8052xylR, 8052xylR-xylT$_{adc}$, 8052xylR-xylT$_{ptb}$ and 8052xylR-xylT$_{thl}$ in XHP2 medium containing 60 g/L xylose. Fermentation were performed in triplicate. Samples were taken at 96 h.

Strain	D-xylose consumption (g/L)	Products (g/L)				Growth (A600)
		Acetone	Butanol	Ethanol	ABE	
8052WT	32.68 ± 0.19	1.27 ± 0.06	5.76 ± 0.00	0.85 ± 0.07	7.88 ± 0.06	3.65 ± 0.30
8052xylR	45.23 ± 0.78	1.96 ± 0.05	9.67 ± 0.27	0.99 ± 0.01	12.61 ± 0.22	6.79 ± 0.60
8052xylR-xylT$_{adc}$	45.34 ± 0.87	2.30 ± 0.18	8.86 ± 0.22	1.03 ± 0.06	12.19 ± 0.32	5.80 ± 0.23
8052xylR-xylT$_{ptb}$	55.84 ± 0.92	2.89 ± 0.28	11.56 ± 0.11	1.43 ± 0.11	15.89 ± 0.22	8.06 ± 0.26
8052xylR-xylT$_{thl}$	46.93 ± 0.89	2.40 ± 0.26	10.17 ± 0.20	1.20 ± 0.12	13.76 ± 0.10	7.41 ± 0.55

Table 3
Transcriptional fold-changes of gene *xylT* in three engineered strains (8052xylR-xylT$_{adc}$, 8052xylR-xylT$_{ptb}$, and 8052xylR-xylT$_{thl}$) compared to the 8052xylR strain.

Strains	Transcriptional level (mean fold change ± SD)	
	Acidogenic phase[a]	Solventogenic phase[a]
8052xylR-xylT$_{adc}$	6.57 ± 0.85	0.87 ± 0.07
8052xylR-xylT$_{ptb}$	4.53 ± 0.69	2.20 ± 0.37
8052xylR-xylT$_{thl}$	2.38 ± 0.39	3.27 ± 0.34

[a] OD_{600} of samples taken in acidogenic and solventogenic phase were around 0.6 and 2.5, respectively. 16S *rRNA* gene (cbeir0001) was used as an internal control. Fermentations were performed in XHP2 medium containing 60 g/L D-xylose as the carbon source.

In bacteria, D-xylose uptake occurs generally through an ATP-binding cassette (ABC) transporter and a proton symporter (Walmsley et al., 1998). Although the proton-linked symporter normally exhibits lower affinity (higher K_M) than the ABC transporter, it was nevertheless an important target to engineer for improving D-xylose uptake (Jojima et al., 2010). Gene cbei0109 of *C. beijerinckii* NCIMB 8052 was preferentially chosen here as the *xylT* candidate to overexpress because, among the annotated 14 sugar transporters, it shared the highest homology with a previously identified *xylT* gene (cac1345) in *C. acetobutylicum* (Gu et al., 2010) (Table S2). Moreover, that nearly 50% less D-xylose consumption by the *xylT* mutant strain (8052xylT) in comparison with that of the 8052WT (results 3.2) does demonstrate the important role cbei0109 plays in D-xylose uptake in *C. beijerinckii*. Of course, it should be noted that a D-xylose ABC transporter was also found in the genomic context of *C. beijerinckii*, namely cbei2380-cbei2382 (Gu et al., 2010). However, we did not preferentially choose this ABC transporter for overexpression because its disruption only caused 5-6% less D-xylose consumption (unpublished data), indicating that this transporter may not dominate in D-xylose uptake in *C. beijerinckii*.

When overexpressing *xylT* gene in the 8052xylR strain, three promoters (*adc*, *ptb* and *thl*) were adopted. Although the transcriptional levels of gene *xylT* (Table 3), driven by these promoters, were not exactly consistent with the β-galactosidase activities from promoter-*lacZ* fusion (Fig. 3), which may be caused by other factors (e.g. mRNA stability and translation efficiency), all three promoters were proven to work in *C. beijerinckii*. In addition, we also noticed the gradually decreased β-Gal activities driven by these three promoters along the fermentation. This should be attributed to the instability of pIMP1 plasmid in *C. beijerinckii* without antibiotic selection pressure. Interestingly, only the *ptb* promoter conferred an obvious advantage to the 8052xylR in D-xylose consumption (Fig. 4), despite that β-galactosidase activity driven by the *ptb* was not the highest among the candidates (Fig. 3). Therefore, it seems that a strong overexpression of *xylT* gene here might not equate to the optimal expression level. Using the promoter with moderate strength for gene overexpression and pathway optimization were also reported in the previous studies (Alper et al., 2005; Bond-Watts et al., 2011; Lu and Jeffries, 2007). Thus, further engineering strategies for a better D-xylose consumption may focus on: (*i*) fine-tuning expression level of the *xylT* gene by artificial promoters (Davis et al., 2011; Hammer et al., 2006); (*ii*) mutating the 5'-untranslated region of the *xylT* to enhance mRNA stability and translation efficiency (Huang et al., 2006; Komarova et al., 2005).

Since D-xylose is the most abundant pentose presented in xylose mother liquid, disruption of the *xylR*-like gene, in combination of a *xylT* gene overexpression, appears to be a successful strategy here to permit efficient utilization of this industrial byproduct by *C. beijerinckii*. An interesting result is that the consumption of L-arabinose, another main "building block" in hemicellulose, by the 824 WT, 824xylR and 8052xylR-xylT$_{ptb}$ gradually increased (Table S4). There is usually another set of genes responsible for L-arabinose uptake and metabolism in microbes. Such as in *E. coli*, there are AraE, that is required for L-arabinose uptake, AraBAD enzymes, that are required for L-arabinose catabolism, and the regulatory protein AraC, (Schleif, 2010). For the solventogenic clostridia, a recent study also confirmed an L-arabinose operon in *C. acetobutylicum* ATCC 824 (Zhang et al., 2012). Considering the role XylT plays in L-arabinose uptake (Gu et al., 2010; Servinsky et al., 2010), we speculated that the phenotypic improvement in L-arabinose consumption of the 8052xylR and 8052xylR-xylT$_{ptb}$ here might be attributed to *xylT* up-regulation (Fig. 1A) after *xylR* inactivation and XylT overexpression, respectively.

We also noticed that L-arabinose utilization still requires further improvements (3.43 g/L remained) for the 8052xylR-xylT$_{ptb}$ (Fig. 5A). Although L-arabinose is not as dominant as D-xylose in the xylose mother liquid, its efficient utilization is still crucial for increasing conversion yield of this renewable material. Similar engineering strategies could be adopted to address this problem in the following work. For example, a possible AraR regulon (cbei4452-cbei4457) can be found in the genome context of *C. beijerinckii*, which contains the transcriptional regulator gene *araR*, L-arabinose isomerase gene *araA*, L-ribulokinase gene *araB* and L-ribulose-5-phosphate 4-epimerase gene *araD*, thereby providing a useful clue for further genetic modification of L-arabinose pathway in *C. beijerinckii*. In addition, it should be pointed out that the xylose mother liquid medium prepared for fermentation in this study was a synthetic medium, and thus, a cheaper medium formulation is still required for future industrial-scale solvent production from xylose mother liquid.

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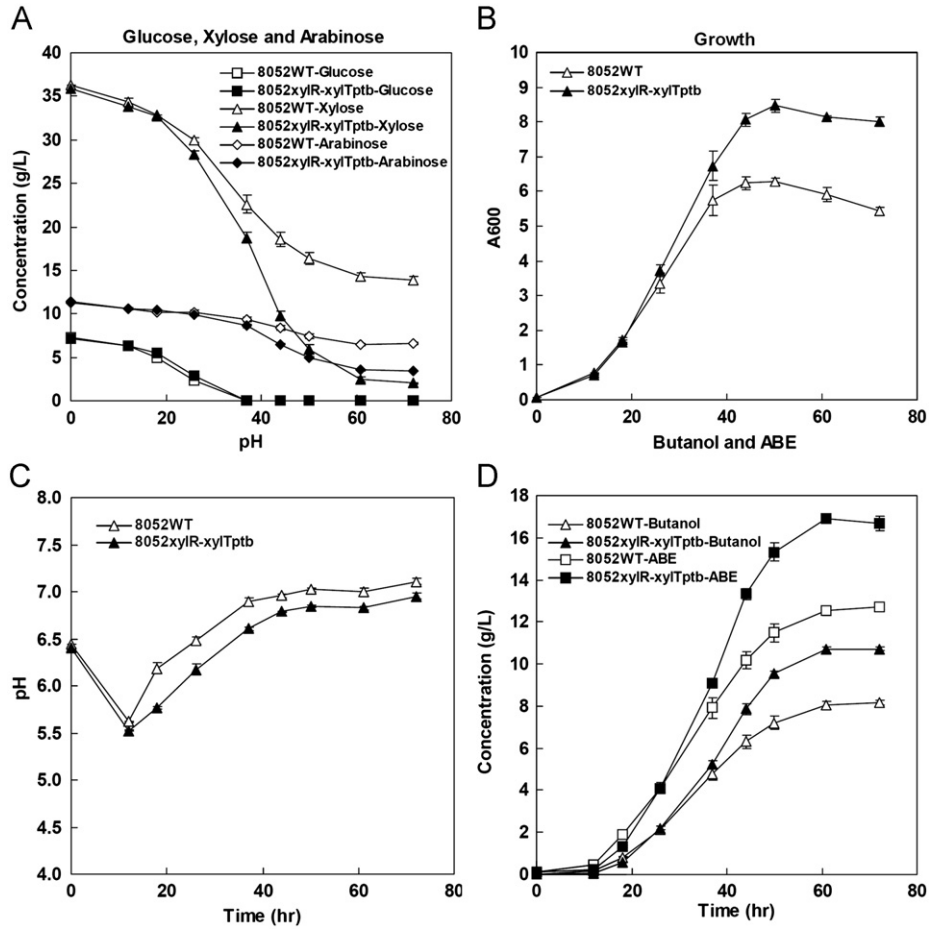

Fig. 5. Growth and metabolite profiles of the 8052WT and 8052xylR-xylT$_{ptb}$ strain in fermenting xylose mother liquid. (A) Sugars consumption; (B) Growth; (C) pH values; (D) Butanol and ABE concentrations. Fermentations were performed in triplicate.

Table S4
Metabolite profiles of the 8052WT, 8052xylR and 8052xylR-xylT$_{ptb}$ strain in fermenting xylose mother liquid. Fermentation were performed in triplicate. Samples were taken at 48 h.

Strains	Residual sugars (g/L)			Products (g/L)				Growth (A600)
	D-Glucose	D-Xylose	L-Arabinose	Acetone	Butanol	Ethanol	Total solvents	
8052WT	0	15.83 ± 0.60	7.84 ± 0.21	2.57 ± 0.10	6.80 ± 0.19	0.41 ± 0.01	9.78 ± 0.02	6.84 ± 0.33
8052xylR	0	6.75 ± 0.67	3.95 ± 0.34	3.49 ± 0.17	10.82 ± 0.24	0.63 ± 0.01	14.94 ± 0.28	8.79 ± 0.53
8052xylR-xylT$_{ptb}$	0	2.47 ± 0.23	2.62 ± 0.22	4.62 ± 0.06	11.27 ± 0.08	0.76 ± 0.05	16.65 ± 0.10	9.38 ± 0.22

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Appendix A Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.diff.2012.05.001.

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