



ELSEVIER

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Selective reduction of xylose to xylitol from a mixture of hemicellulosic sugars

Nikhil U. Nair^a, Huimin Zhao^{a,b,*}

^a Department of Chemical and Biomolecular Engineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^b Departments of Chemistry, Biochemistry, Bioengineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

ARTICLE INFO

Article history:

Received 28 January 2010

Received in revised form

24 March 2010

Accepted 26 April 2010

Available online 4 May 2010

Keywords:

Xylitol

Arabinitol

Xylose reductase

Selectivity

Biocatalysis

Hemicellulose

ABSTRACT

The biocatalytic reduction of D-xylose to xylitol requires separation of the substrate from L-arabinose, another major component of hemicellulosic hydrolysate. This step is necessitated by the innate promiscuity of xylose reductases, which can efficiently reduce L-arabinose to L-arabinitol, an unwanted byproduct. Unfortunately, due to the epimeric nature of D-xylose and L-arabinose, separation can be difficult, leading to high production costs. To overcome this issue, we engineered an *E. coli* strain to efficiently produce xylitol from D-xylose with minimal production of L-arabinitol byproduct. By combining this strain with a previously engineered xylose reductase mutant, we were able to eliminate L-arabinitol formation and produce xylitol to near 100% purity from an equiweight mixture of D-xylose, L-arabinose, and D-glucose.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Xylitol has several favorable properties as a sugar substitute, such as low caloric content (Parajo et al., 1998), anticariogenicity (Edgar, 1998; Roberts et al., 2002), good gastrointestinal tolerance, and near insulin-independent metabolism in humans. The traditional production of xylitol involves direct chemical hydrogenation of hemicellulosic hydrolysates over a Raney-Nickel catalyst followed by extensive purification from non-specific reduction products. Biocatalytic routes to xylitol production using fungal or yeast xylose reductase (XR) have also been explored (Bae et al., 2004; Kim et al., 2004; Parajo et al., 1998; Silva et al., 2003). Unfortunately, the non-specific nature of direct hydrogenation is only partially addressed in the biocatalytic route. The natural promiscuity of XRs toward other sugars, particularly L-arabinose, another major component of hemicelluloses, necessitates the prior purification of D-xylose to minimize formation of L-arabinitol. Since D-xylose and L-arabinose are epimers, their separation is non-trivial, and is one of the leading obstacles to the more economical production of xylitol.

We had previously isolated an XR from the filamentous fungus *Neurospora crassa*, which has an innate 2.4-fold preference for

D-xylose over L-arabinose (Woodyer et al., 2005). Resting cell studies in recombinant *E. coli* expressing this enzyme demonstrated that such a small difference in selectivity is sufficient to improve the ratio of xylitol-to-arabinitol produced (Nair and Zhao, 2008). To increase the selectivity of the process toward xylitol, we engineered the XR for decreased L-arabinose reductase activity, and via several rounds of directed evolution found a mutant VMQCI that had a 50-fold lower catalytic efficiency toward L-arabinose (Nair and Zhao, 2008). Resting cell studies with this mutant revealed that although the amount of L-arabinitol was significantly decreased, it was not completely eliminated. In order to further increase the selectivity of this biocatalytic process, we decided to implement an orthogonal strategy to reduce final L-arabinitol titer. By combining the previously engineered protein with a metabolic engineering strategy—a combination that has not been fully explored to create biocatalysts with novel properties, we hope to exploit the possible synergy between the two approaches.

To realize this goal, we needed to create a metabolically engineered *E. coli* strain that is highly efficient at utilizing L-arabinose as a carbon source, and able to sequester it away from XR, decreasing L-arabinitol production. One of the major obstacles to create such a strain is that the regulation of various catabolic pathways of *E. coli* in the presence of multiple sugars is not well understood. This is particularly important for selective production of xylitol from hemicellulosic hydrolysate since corn fiber consists of D-xylose, L-arabinose, and D-glucose. While diauxic growth pattern due to glucose repression in *E. coli* is well

* Corresponding author at: Department of Chemical and Biomolecular Engineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. Fax: +1 217 333 5052.

E-mail address: zhao5@illinois.edu (H. Zhao).

studied, little is known about the relative preference between pentoses, and even less in the presence of glucose. In addition, the system we have been using so far to overexpress our XR is IPTG (isopropyl- β -D-thiogalactopyranoside)-dependent, which is reliant on the lactose system, thus introducing a fourth regulatory system. Considering that the metabolism of all three non-glucose sugars is dependent on activation by CRP (cyclic adenosine monophosphate receptor protein), significant cross-talk between them is to be expected. Glucose de-repression for simultaneous uptake of two sugars has been documented previously, albeit primarily for ethanol production, which was carried out under oxygen-limited conditions (Grotkjaer et al., 2005; Lindsay et al., 1995; Nichols et al., 2001). The pleiotropic effects on other regulatory systems of such de-repressed mutants are poorly characterized.

To engineer *E. coli* for efficient L-arabinose catabolism in the presence of glucose and D-xylose, we tested three different de-repressed strains—a glucose phosphotransferase mutant, a regulation deficient adenylate cyclase mutant, and a CRP mutant (Goerke and Stulke, 2008). We found that the *crp** mutant is superior among the three. This mutant was previously described to be helpful in co-utilization of D-xylose and glucose for the production of xylitol using an IPTG induction system (Cirino et al., 2006). We tested the effects of overexpressing XR under the control of D-xylose-, IPTG-, and L-arabinose-inducible systems, as well as those of expressing the xylose transporter (XylE). Results indicate that under certain conditions, L-arabinose was preferred over glucose, whereas under other growth conditions glucose was the preferred carbon source. Finally, we show that in a bioreactor setting, our engineered strain in conjunction with the mutant XR (VMQCI) is able to eliminate L-arabinol production from an equiweight mixture of D-xylose, L-arabinose, and glucose.

2. Materials and methods

2.1. Materials

All media were purchased from Becton-Dickinson (BD, Sparks, MD), chemicals from Sigma-Aldrich (St. Louis, MO), enzymes from New England Biolabs (NEB, Beverly, MA), and oligonucleotide primers from Integrated DNA Technologies (IDT, Coralville, IA). All DNA purification kits were obtained from Qiagen (Valencia, CA), except that the Wizard[®] Genomic DNA Purification Kit was procured from Promega (Madison, WI). Cells were maintained on Lysogeny Broth (LB) plates containing 1.5% agar and the appropriate antibiotic. Selection for plasmid maintenance was done with ampicillin (100 mg/L), chloramphenicol (25 mg/L), and kanamycin (50 mg/L). Chromosomal integrants were selected on chloramphenicol (6 mg/L) or tetracycline (10 mg/L) LB plates.

2.2. Plasmid construction

All cloning work was performed in *E. coli* DH5 α or WM1788 (*pir*⁺ for propagating R6 K plasmids), and a list of constructs can be found in Table 1. All XR expression plasmids were derivatives of pTrc99A. XR and mutants were previously cloned into pACYCDuet (Novagen), and were used as the template for PCR (Nair and Zhao, 2008). The XylA promoter was amplified from *E. coli* MG1655 genomic DNA, and spliced with XR using overlap extension PCR. The cassette was digested with NsiI and BglII and ligated into pTrc99A that had been digested with NsiI and BamHI. Ligation of compatible BglII-BamHI ends abolished both restriction sites. The AraBAD promoter was digested out of pRW2-ptdh (Johannes et al., 2005) using PstI and NdeI; PCR amplified XR was digested with

Table 1
Strains and plasmids used in this study.

Name	Relevant characteristics	Source
<i>Plasmids</i>		
pTrc99A	Amp, pBR322-derived plasmid	Amersham Pharmacia Novagen
pACYCDuet pACYC-ncxr	Cm, p15A-derived plasmid template for XR	Nair and Zhao, 2008
pACYC-VMQCI	template for XR mutant VMQCI	Nair and Zhao, 2008
pTKXb-xdharab'	Km, Source of BLMA promoter	Nair and Zhao, 2008
pRW2-ptdh	Km, Source of AraBAD promoter	Johannes et al., 2005
pXXR	pTrc99A with XR under XylA promoter	This work
pXVMQCI	pTrc99A with VMQCI under XylA promoter	This work
pAraXR	pTrc99A with XR under AraBAD promoter	This work
pAraVMQCI	pTrc99A with VMQCI under AraBAD promoter	This work
pTrcXR	pTrc99A with XR under Trc promoter	This work
pTrcVMQCI	pTrc99A with VMQCI under Trc promoter	This work
pACYCBLMAXylE	pACYCDuet with xylE under BLMA promoter	This work
pACYCAraXylE	pACYCDuet with xylE under AraBAD promoter	This work
<i>Strains</i>		
MG1655	gDNA template for XylA promoter and xylE	ATCC 700926
C600	<i>F</i> ⁻ <i>tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ</i> ⁻	CGSC, Yale University
ET23	source of <i>crp*</i> :: <i>Tn10</i>	Eppler and Boos, 1999
HZ1302	C600 <i>crp*</i> :: <i>Tn10</i>	This work
HZ1743	C600 Δ <i>ptsG</i> :: <i>FRT</i>	This work
HZ1651	C600 Δ <i>cyaA</i> ^{regul} :: <i>cat</i>	This work
HZ1450	HZ1302 Δ <i>xylA</i> :: <i>FRT</i>	This work
HZ1967	HZ1302 Δ <i>xylAB</i> :: <i>FRT</i>	This work
HZ1756	HZ1743 Δ <i>xylA</i> :: <i>FRT</i>	This work
HZ1434	HZ1450 with pXXR	This work
HZ1435	HZ1450 with pXVMQCI	This work
HZ1757	HZ1756 with pXXR	This work
HZ2008	HZ1450 with pXXR, pACYCAraXylE	This work
HZ2009	HZ1450 with pXXR, pACYCBLMAXylE	This work
HZ2046	HZ1967 with pTrcXR, pACYCAraXylE	This work
HZ2061	HZ1967 with pAraXR, pACYCAraXylE	This work
HZ2062	HZ1967 with pAraVMQCI, pACYCAraXylE	This work

NdeI and BglII, and pTrc99A with NsiI and BamHI. All three were ligated together in a single reaction, which abolished the compatible PstI-NsiI and BglII-BamHI sites. For IPTG inducible constructs, XR (EcoRI-BglII) was directly ligated into EcoRI-BamHI digested pTrc99A. Xylose transporter *xylE* was amplified from MG1655 genomic DNA and ligated directly into pTKXb-xdh-araB' (Kim et al., 2003; Nair and Zhao, 2008) digested with NdeI and XhoI. The promoter-gene cassette was then digested out with EcoRI and XhoI and ligated in pACYCDuet digested with the same endonucleases. This construct provided expression from the constitutive BLMA promoter. For expression under the AraBAD promoter, *xylE* was first cloned into pRW2-ptdh between the NdeI and PciI sites. The promoter-gene cassette was then digested out using PstI and PciI and ligated into pACYCDuet digested with PstI and NcoI. The ligation abolished the compatible NcoI-PciI sites.

2.3. Genetic methods

All strains used for xylitol production were *E. coli* K-12 C600 and its derivatives (Table 1), and all deletions were performed using

the λ red system (Datsenko and Wanner, 2000). Briefly, PCR product containing the *cat* gene flanked by FRT (Flp recognition target) and 45–50 nt of sequence identical to the target locus was transformed into cells expressing λ red recombinase proteins (encoded on pKD46). Gene replacement was selected on chloramphenicol plates and verified by functional assay and PCR. The resistance marker was then removed by the expression of Flp recombinases from a thermo-inducible promoter on a temperature sensitive plasmid (pCP20). Flp recombinase plasmid loss and *cat* loss occurred simultaneously and were verified by sensitivity to ampicillin and chloramphenicol. Deletion of *ptsG* and *cyaA*^{regul} was performed directly in C600, whereas inactivation of the *xylA* and *xylAB* genes was performed in MG1655 and then moved by P1 transduction to the recipient strains (Miller, 1992). The *crp*^{*} mutation was also generated by P1 transduction from ET23 and selecting for Tet^R integrants (Eppler and Boos, 1999). Deletions were verified by PCR using cell lysate as the template and appropriate flanking primers. Verification of glucose de-repression was first done by blue/white screening on LB plates containing 10 g/L glucose. Strong induction of *lacZ* in the presence of glucose indicated the depressed phenotype. The *CyaA* mutant strain did not demonstrate significant *LacZ* activity. Finally, direct monitoring of sugar co-utilization in shake flasks was used to verify de-repression.

2.4. HPLC analysis

Sugar concentrations were quantified using Shimadzu high performance liquid chromatography (HPLC) equipped with a low temperature evaporative light scattering detector (ELSD-LT) (Columbia, MD). A Bio-Rad Aminex 250 \times 4 mm HPX-87C (Bio-Rad, Hercules, CA) carbohydrate column was used to separate the sugars, as per manufacturer's recommendations. The column was run at 0.2 mL/min at 85 °C for 18 min with water as the mobile phase.

2.5. GC-MS analysis

Acetate quantification was performed at the Roy J. Carver Metabolomics Center. *n*-Butanol (1 mL/L) was used as internal standard to quantify acetate in media. Samples (1 μ L) were injected in split mode (5:1) to the GC/MS system consisting of an Agilent 7890 gas chromatography, an Agilent 5975 mass selective detector, and HP 7683B autosampler (Agilent Technologies, Palo Alto, CA). Acetate samples were analyzed on a 30 m ZB-Wax-Plus column with 0.32 mm I.D. and 0.25 μ m film thickness (Phenomenex, Torrance, CA) with an injection port temperature of 250 °C, the interface set to 250 °C, and the ion source adjusted to 230 °C. The helium carrier gas was set at a constant flow rate of 2.5 mL/min. The temperature program was 5 min isothermal heating at 90 °C, followed by an oven temperature increase of 10 °C/min to 210 °C for 2 min. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in *m/z* 50–550 scan range.

The spectra of all chromatogram peaks evaluated using the HP Chemstation program (Agilent Technologies, Palo Alto, CA). Identification was performed using the mass spectra obtained from the authentic standards and additionally confirmed with NIST08 and W8N08 libraries.

2.6. Shake flask and bioreactor cultures

For shake flask cultures, overnight cultures were grown at 37 °C in M9 minimal medium supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 20 mg/L leucine, 120 mg/L threonine, 10 mg/L

thiamine-HCl, 2 g/L glucose and the appropriate antibiotic(s). 125 mL unbaffled bottles containing 25 mL of the same medium but containing 1–2 g/L of each sugar (glucose, D-xylose, and L-arabinose) were placed under vacuum, filled with nitrogen, and capped with airtight stoppers to maintain oxygen-limited conditions. About 1 mL of overnight cultures were inoculated into these bottles and maintained at 30 °C or 37 °C at 250 rpm. For bioreactor studies, 4 mL overnight cultures were grown at 37 °C either in LB or M9 medium supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 20 g/L glucose, 10 g/L tryptone, and the appropriate antibiotic(s). Upon reaching saturation, these cultures were spun down and resuspended in 4 mL of the same medium and cultured for another 4 hours. These cultures were then inoculated into 400 mL bioreactors containing the same M9+tryptone medium with additional 20 g/L each of D-xylose and L-arabinose, as well as antifoam agents. Bioreactors were run at 30 °C with 400 rpm agitation and 0.8 L/min sparging with air. pH was maintained at 7.0 \pm 0.1 with 5 N NaOH and 2 N H₂SO₄.

3. Results and discussion

3.1. The *crp*^{*} mutant is the most efficient at co-utilizing three sugars for xylitol production

We tested three different catabolite de-repressed mutants—HZ1743, HZ1651 and HZ1302 (Δ *ptsG*, Δ *cyaA*^{reg}, and *crp*^{*}, respectively) for co-utilization of glucose, D-xylose, and L-arabinose. The phosphotransferase system (PTS) for simultaneous glucose uptake and phosphorylation has been shown to play a role in catabolite repression (Goerke and Stulke, 2008). Strains with inactivated permease, *PtsG*, were shown to relieve said repression and have been used for co-fermenting mixed sugars (Nichols et al., 2001). Adenylate cyclase (*CyaA*) is responsible for forming cAMP in response to low glucose concentrations. Its activity is regulated by interaction with the PTS protein Enzyme IIA^{Glc}. A strain with truncated *CyaA* was shown to be de-regulated and did not demonstrate diauxic behavior when grown in glucose and maltose mixtures (Crasnier et al., 1994). Several CRP (also known as CAP, catabolite activator protein) mutants have been isolated that show de-repressed behavior (Eppler and Boos, 1999; Karimova et al., 2004; Zhu and Lin, 1988). For this work, we picked the CRP mutant that was shown to de-repress xylose metabolism under aerobic condition for xylitol production (Cirino et al., 2006; Eppler and Boos, 1999).

For this study, deletions were created by replacing the undesired locus with PCR amplified *cat* (Cm^R) mediated by λ red recombinase proteins (Datsenko and Wanner, 2000), either directly in the parent strain, or in MG1655 and then transduced into the appropriate recipient (Miller, 1992). The CRP mutant was created by transduction of donor allele from ET23 into C600 (Eppler and Boos, 1999; Miller, 1992).

These three recombinant strains plus the wild-type strain were grown in minimal medium with \sim 2 g/L each of glucose, D-xylose, and L-arabinose under oxygen-limited conditions. Supernatants were analyzed at various time points to ascertain their sugar utilization patterns (Fig. 1). The wild-type C600 demonstrated strong diauxic, with almost no uptake of D-xylose or L-arabinose until complete depletion of glucose. The strain with truncated *CyaA* (HZ1651) showed slightly decreased glucose assimilation, although pentose utilization was not significantly improved. The *PtsG* knockout (HZ1743) demonstrated delayed response to glucose, as expected (Nichols et al., 2001), but was able to uptake L-arabinose and glucose simultaneously, albeit with differing rates. Finally, the *crp*^{*} mutant (HZ1302) showed efficient simultaneous assimilation of all three sugars, although, as in all

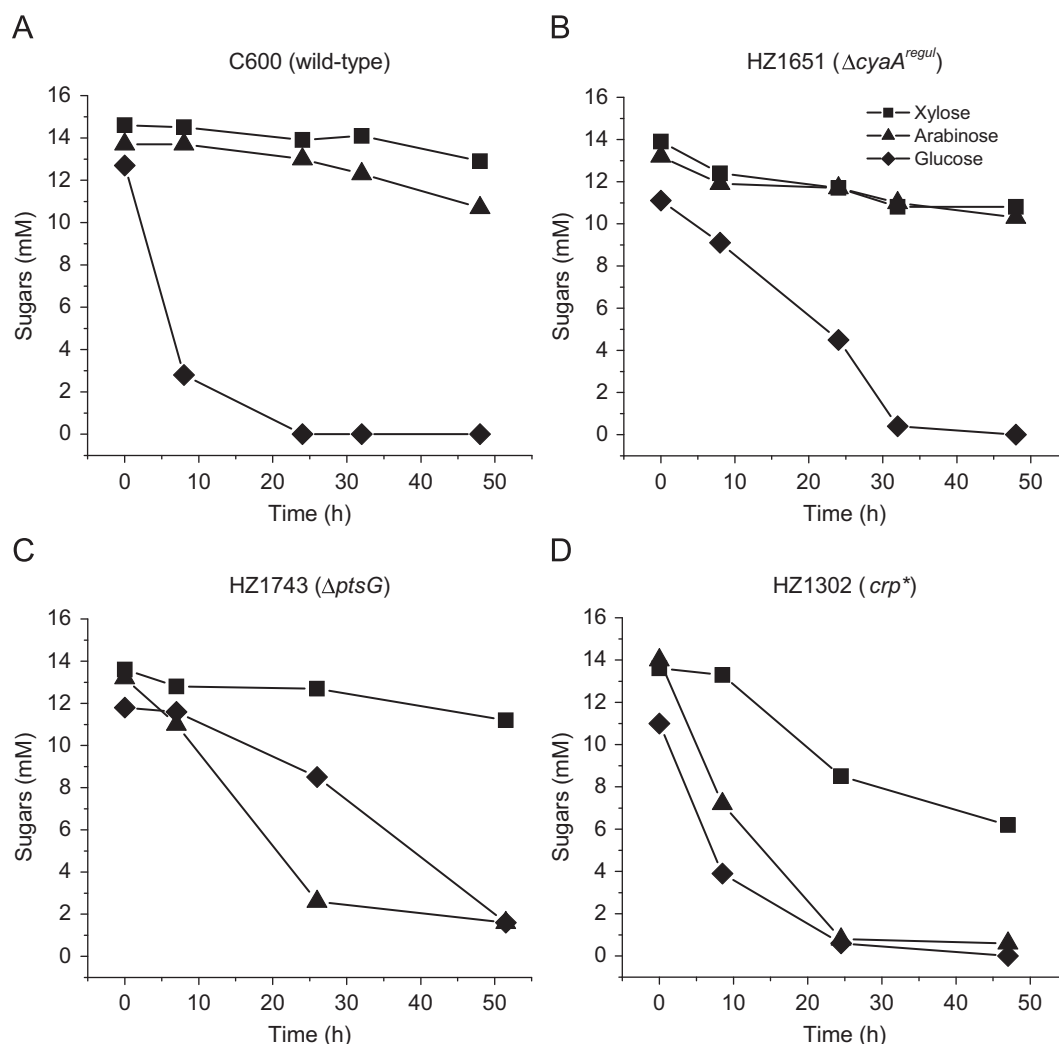


Fig. 1. Growth of various strains in glucose, D-xylose, and L-arabinose to test for catabolite repression at 30 °C. (A) Wild-type *E. coli* K-12 C600 shows strong diauxie, with quick utilization of glucose first. (B) Deletion of the regulatory domain of adenylate cyclase (HZ1651, $\Delta cyaA^{regul}$) resulted in slightly less pronounced diauxie, although pentose assimilation is still slower than glucose. (C) Glucose permease knockout (HZ1743, $\Delta ptsG$) strain showed efficient L-arabinose and glucose utilization, although D-xylose was relatively slower. (D) The mutant CRP (HZ1302, crp^*) showed the most efficient co-utilization of all three sugars. All experiments were also performed at 37 °C to ascertain glucose de-repressed phenotype.

strains, xylose uptake was the slowest. Based on these data, HZ1651 was deemed unsuitable for xylitol production. After deletion of *xyIA* in HZ1743 and HZ1302 to prevent xylose catabolism, pXXR (wtXR under XylA promoter) was transformed into both strains to give HZ1757 and HZ1434, respectively, and tested for xylitol productivity (Fig. 2). Although both strains demonstrate efficient utilization of glucose and L-arabinose as carbon source, the stronger induction from xylose promoters in HZ1434 is evident from higher xylose conversion to xylitol. Based on these experiments, the crp^* mutant strain was used for further engineering work.

3.2. Crabtree effect is prevalent at high sugar concentrations in the crp^* strain

Glycolysis rate at high sugar concentrations often exceeds respiratory capacity, leading to build up of intermediate metabolites. This “Crabtree effect” is well-known for many organisms including *S. cerevisiae* and *E. coli*, which are known to build up ethanol and acetate, respectively. In *E. coli* acetate build-up decreases growth rate as well as recombinant protein production (Eiteman and Altman, 2006). Previous work in a similar crp^* strain showed that at 18 g/L glucose concentration,

acetate production is significant, accumulating to 70 mM (Akin-terinwa and Cirino, 2009).

When HZ1434 was grown in 40 g/L total usable sugar (glucose+L-arabinose) in minimal M9 medium, we found that pH dropped to ~5 within 24 h, completely inhibiting growth due to high level acetate production (Fig. 3). Addition of 50 mM MOPS (4-morpholinopropanesulfonic acid) to the medium could not buffer the pH at 7.0, as had been done previously at 18 g/L glucose (Cirino et al., 2006). Addition of a complex nitrogen source has been shown to reduce acetate production in batch cultures (Panda et al., 2000). However, addition of 10 g/L tryptone did not prevent acid accumulation. Although genetic methods exist to decrease acetate production (De Anda et al., 2006), pleiotropic effects could lead to additional complications. Therefore, we chose to use a pH-stat bioreactor for further studies.

3.3. Expression from arabinose promoter decreases Crabtree effect and lag phase

In the pH-stat bioreactor with 60 g/L total sugars (equiweight D-xylose, L-arabinose, and glucose), there was a ~24 h lag phase.

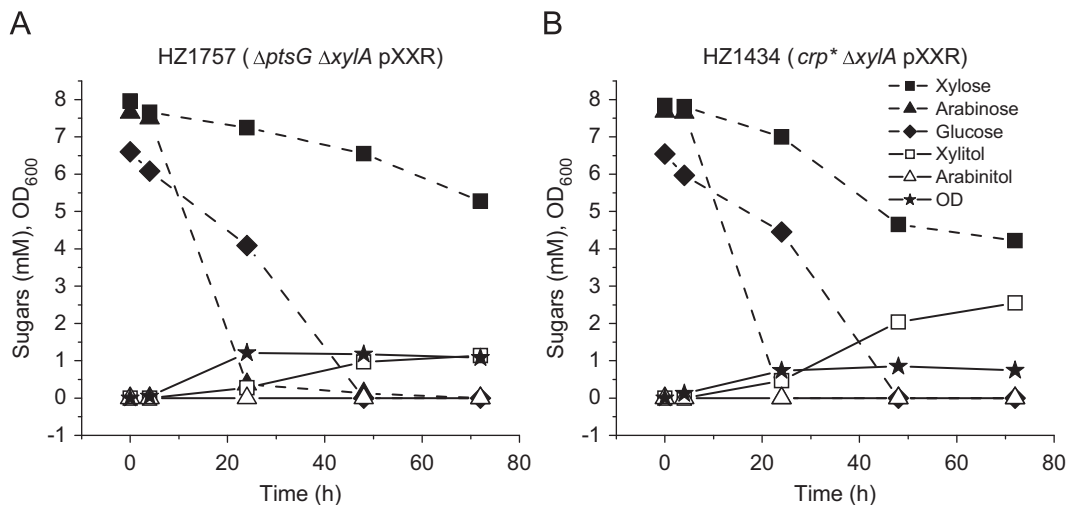


Fig. 2. Xylitol production in shake flasks comparing (A) HZ1757 ($\Delta ptsG \Delta xylA$ pXXR) and (B) HZ1434 ($crp^* \Delta xylA$ pXXR) diauxic relief strategies. Although both strains demonstrate simultaneous glucose and L-arabinose assimilation, stronger induction of the xylose pathway results in higher xylitol production using XR under XylA promoter in HZ1434. Neither of the two strains produces significant amounts of L-arabinitol. Data are an average of two independent experiments and error is less than 15% in all cases. Experiments were also performed with mutant VMQCI, and similar results were obtained.

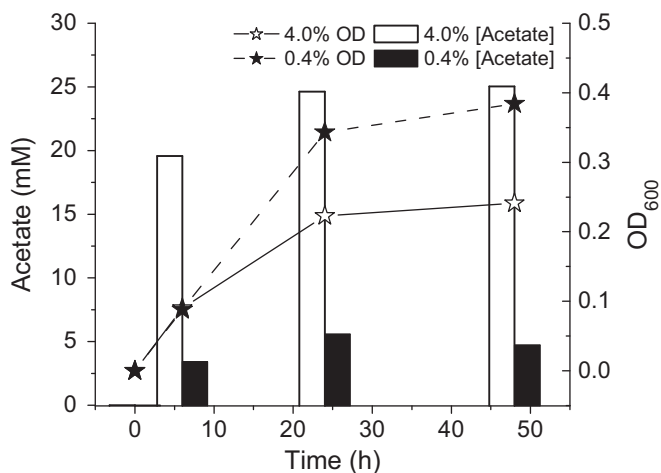


Fig. 3. Acetate production by HZ1434 during growth in 4% and 0.4% usable sugars (glucose+L-arabinose). Cells grown in high concentrations of sugars succumb to Crabtree effect and produce large amounts of acetate (~25 mM), which inhibits cell growth, resulting in decreased final cell density. Data points are shown at 0, 6, 24, 48 h, and are an average of two independent experiments and error is less than 15% in all cases.

In addition, xylitol production was minimal until near-complete depletion of L-arabinose in the medium (Fig. 4A). We reasoned that poor induction of the xylose pathway compared to the arabinose operon (Fig. 1D) was the primary reason for low productivity. Since overexpression of xylose-proton symporter (XylE) was shown to transport D-xylose efficiently in glucose-xylose mixtures (Khankal et al., 2008), we reasoned it may help increase xylitol productivity. Expression using a constitutive promoter, BLMAp (Kim et al., 2003) using pACYCBLMAXylE in HZ2009 (Table 1), did not improve xylitol conversion (data not shown). On the other hand, expression of XylE under the AraBAD promoter from a multicopy plasmid (pACYCAraXylE) had the unexpected side-effect of simultaneously decreasing both the lag phase of HZ2008 and the total amount of alkali required to maintain pH at 7.0 (Fig. 4B). Unfortunately, the xylitol productivity was nearly unaltered. Another side-effect of this is the change of the relative rates of glucose and L-arabinose consumption. Prior

to XylE overexpression (HZ1434), L-arabinose was assimilated faster than glucose (Fig. 2B and Fig. 4A), whereas after its overexpression (HZ2008), glucose was the preferred carbon source (Fig. 4B). It is possible that promoter dilution may play a role in decreasing expression from the chromosomal *araBAD* operon, although previous reports indicate that this phenomenon is not significant in bacteria (Forde et al., 2004) or yeast (Bae et al., 2008). Alternately, the presence of XylE in the cell membrane either replacing AraE and AraGFH transporters, or in addition to them, could be retarding the rate of L-arabinose uptake. This could also explain the lower requirement for alkali in the bioreactor, since the respiration rate would be more capable of keeping up with the slower glycolysis of L-arabinose. The reason behind the decreased lag phase is difficult to decipher from these experiments, and will probably require a more thorough characterization of the HZ1967 and HZ2008 transcriptome, proteome, and metabolome.

Since overexpression of XylE did not improve the final xylitol titer, we reasoned the poor productivity was due to low expression of XR under the control of XylA promoter. So, we placed XR under either the IPTG-inducible Trc promoter (pTrcXR) or the AraBAD promoter (pAraXR). Induction from a *lac*-based promoter in *crp^** strain in glucose-xylose mixtures was previously shown to produce high levels of recombinant protein, even at 100 μ M concentration (Cirino et al., 2006). However, expression of XR from the Trc promoter induced with 100 μ M IPTG led to even poorer conversion than that obtained using the XylA promoter (HZ2046, data not shown). Under the AraBAD promoter (HZ2061), xylitol production reached near-stoichiometric levels, with low levels of L-arabinitol production as well (2–6 mM, Fig. 4C). The VMQCI mutant produced xylitol at a slightly slower rate than wtXR (HZ2062), as would be expected from the lower overall activity of the mutant (Fig. 4D) (Nair and Zhao, 2008), but it produced undetectable levels of L-arabinitol over the 4 day period (limit of detection < 1 mM).

3.4. Catabolic pathways: activation and competition

Catabolic pathways for sugars other than glucose are normally repressed in its presence. We tested three different strategies for

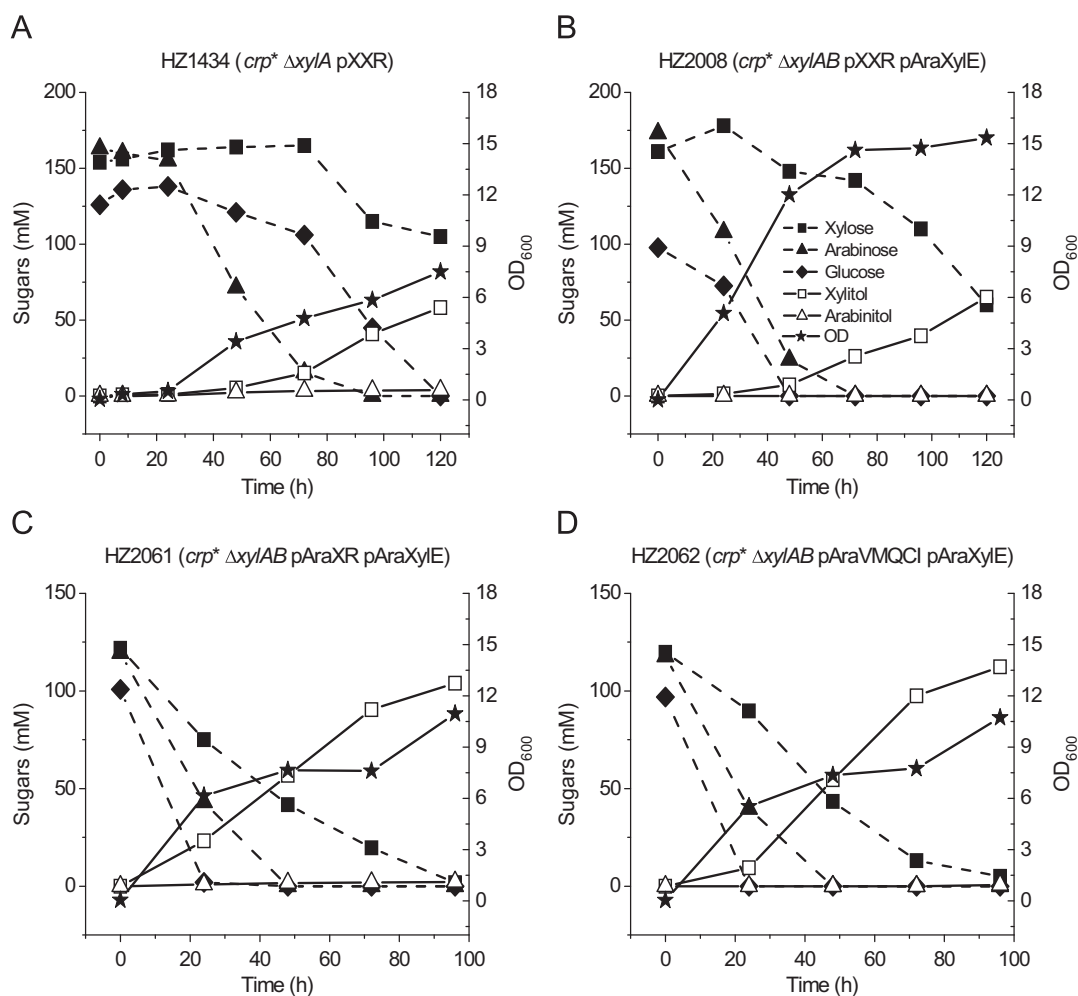


Fig. 4. Strategies implemented to improve xylitol productivity. (A) pH-stat bioreactor allows cells to completely and efficiently catabolize *L*-arabinose and glucose simultaneously. XR expression is under control of the *XylA* promoter (HZ1434). (B) Concurrent expression of xylose-proton symporter (*XylE*) using *AraBAD* promoter decreases lag phase, but also decreases *L*-arabinose assimilation rate relative to glucose (HZ2008). Xylitol productivity does not increase significantly, however. (C) Expression of XR using *AraBAD* promoter instead of *XylA* promoter promotes near-stoichiometric conversion of *D*-xylose to xylitol (HZ2061). (D) Expression of the mutant XR, VMQCI, eliminates *L*-arabinitol production, although initial xylitol productivity also drops slightly (HZ2062). Data are an average of two independent experiments and error is less than 15% in all cases.

de-repression and found that *crp** mutant was the most efficient at simultaneously activating the *D*-xylose and *L*-arabinose metabolic pathways (Fig. 1). However, the arabinose pathway was more strongly activated, as evident from quicker uptake and assimilation compared to *D*-xylose. Using XR as a reporter under the control of arabinose (*AraBAD*), xylose (*XylA*), or lactose (*Trc*) promoter systems, we found again that *AraBAD* was the most strongly expressed among all three. Although the *lac*-based system was shown to be fully activatable with 100 μ M IPTG in *crp** strains in the presence of glucose and *D*-xylose (Cirino et al., 2006), in the presence of three sugars, this promoter was weakly induced. This is true even in light of the fact that IPTG is the only non-transformable inducer tested. In a non-*crp** strain, we have previously seen strong activation of *D*-xylose, *L*-arabinose, and lactose operons simultaneously in the absence of glucose (Nair and Zhao, 2008), and Lee and coworkers have shown that presence of IPTG represses *AraBAD* promoter activation (Lee et al., 2007). In contrast to these observations, in the *crp** strain created here, we found the exact opposite—*L*-arabinose repressed activation from IPTG-dependent promoters. Investigations into the mechanism of competition and cross-talk between the regulation of three non-glucose

operons in wild-type and *crp** strains in the presence or absence of glucose would help explain the behavior seen here. The roles of sugar-specific transporters and transcription activators/repressors, in particular, would reveal the mechanism of these interactions.

In conclusion, we have shown that the combination of protein engineering and metabolic engineering can lead to synergistic increase in desired biocatalytic properties. In this particular case, the synergy manifested as increased selectivity such that that *L*-arabinitol production was almost completely eliminated from an equiweight mixture of *D*-xylose, *L*-arabinose, and glucose—the three major sugars in hemicellulosic hydrolysate. Considering actual corn hemicellulose has *D*-xylose-to-*L*-arabinose ratio of \sim 5:3 (Saha, 2003), the tested equiweight mixture is a worst-case scenario. This strategy used an engineered *E. coli* strain with glucose de-repressed growth and xylose transporter overexpression to quickly assimilate *L*-arabinose as a carbon source, sequestering it away from the substrate selective XR mutant VMQCI. Not only is *L*-arabinose prevented from being converted to *L*-arabinitol, it also provides reducing equivalents in the form of NADPH for xylitol production, and acts as an inducer for protein expression.

Acknowledgments

We would like to thank Dr. Ryan P. Sullivan, Fei Wen, and Dr. Byoungjin Kim for their assistance with HPLC analysis and bioreactor studies, as well as Dr. Alexander Ulanov for developing acetate detection protocol. We would also like to acknowledge Prof. Patrick C. Cirino (Pennsylvania State University, PA) for providing *crp** parent strain ET23, Prof. William W. Metcalf (UIUC) for the *pir** cloning strain WM1788, and Prof. John E. Cronan (UIUC) for P1*vir* phage used for transduction. N.U.N. also acknowledges Drickamer Fellowship support from the Department of Chemical and Biomolecular Engineering at the University of Illinois. This work was funded by a subcontract of DE-FG02-07ER84793 (zuChem) and the National Science Foundation as part of the Center for Enabling New Technologies through Catalysis (CENTC), CHE-0650456.

References

- Akinterinwa, O., Cirino, P.C., 2009. Heterologous expression of D-xylulokinase from *Pichia stipitis* enables high levels of xylitol production by engineered *Escherichia coli* growing on xylose. *Metab. Eng.* 11, 48–55.
- Bae, J.Y., Laplaza, J., Jeffries, T.W., 2008. Effects of gene orientation and use of multiple promoters on the expression of *XYL1* and *XYL2* in *Saccharomyces cerevisiae*. *Appl. Biochem. Biotechnol.* 145, 69–78.
- Bae, S.M., Park, Y.C., Lee, T.H., Kweon, D.H., Choi, J.H., Kim, S.K., Ryu, Y.W., Seo, J.H., 2004. Production of xylitol by recombinant *Saccharomyces cerevisiae* containing xylose reductase gene in repeated fed-batch and cell-recycle fermentations. *Enzym. Microb. Tech.* 35, 545–549.
- Cirino, P.C., Chin, J.W., Ingram, L.O., 2006. Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. *Biotechnol. Bioeng.* 95, 1167–1176.
- Crasnier, M., Dumay, V., Danchin, A., 1994. The catalytic domain of *Escherichia coli* K-12 adenylate cyclase as revealed by deletion analysis of the *cya* gene. *Mol. Gen. Genet.* 243, 409–416.
- Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA.* 97, 6640–6645.
- De Anda, R., Lara, A.R., Hernandez, V., Hernandez-Montalvo, V., Gosset, G., Bolivar, F., Ramirez, O.T., 2006. Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of *Escherichia coli* for recombinant protein production without impairment of growth rate. *Metab. Eng.* 8, 281–290.
- Edgar, W.M., 1998. Sugar substitutes, chewing gum and dental caries—a review. *Br. Dent. J.* 184, 29–32.
- Eiteman, M.A., Altman, E., 2006. Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends Biotechnol.* 24, 530–536.
- Eppler, T., Boos, W., 1999. Glycerol-3-phosphate-mediated repression of *maltT* in *Escherichia coli* does not require metabolism, depends on enzyme IIA(Glc) and is mediated by cAMP levels. *Mol. Microbiol.* 33, 1221–1231.
- Forde, C.E., Rocco, J.M., Fitch, J.P., McCutchen-Maloney, S.L., 2004. Real-time characterization of virulence factor expression in *Yersinia pestis* using a GFP reporter system. *Biochem. Biophys. Res. Commun.* 324, 795–800.
- Goerke, B., Stulke, J., 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* 6, 613–624.
- Grottkjaer, T., Christakopoulos, P., Nielsen, J., Olsson, L., 2005. Comparative metabolic network analysis of two xylose fermenting recombinant *Saccharomyces cerevisiae* strains. *Metab. Eng.* 7, 437–444.
- Johannes, T.W., Woodyer, R.D., Zhao, H.M., 2005. Directed evolution of a thermostable phosphite dehydrogenase for NAD(P)H regeneration. *Appl. Environ. Microbiol.* 71, 5728–5734.
- Karimova, G., Ladant, D., Ullmann, A., 2004. Relief of catabolite repression in a cAMP-independent catabolite gene activator mutant of *Escherichia coli*. *Res. Microbiol.* 155, 76–79.
- Khankal, R., Chin, J.W., Cirino, P.C., 2008. Role of xylose transporters in xylitol production from engineered *Escherichia coli*. *J. Biotechnol.* 134, 246–252.
- Kim, T.B., Lee, Y.J., Kim, P., Kim, C.S., Oh, D.K., 2004. Increased xylitol production rate during long-term cell recycle fermentation of *Candida tropicalis*. *Biotechnol. Lett.* 26, 623–627.
- Kim, Y.W., Choi, J.H., Kim, J.W., Park, C., Kim, J.W., Cha, H.J., Lee, S.B., Oh, B.H., Moon, T.W., Park, K.H., 2003. Directed evolution of *Thermus maltogenic* amylase toward enhanced thermal resistance. *Appl. Environ. Microbiol.* 69, 4866–4874.
- Lee, S.K., Chou, H.H., Pflieger, B.F., Newman, J.D., Yoshikuni, Y., Keasling, J.D., 2007. Directed evolution of AraC for improved compatibility of arabinose- and lactose-inducible promoters. *Appl. Environ. Microbiol.* 73, 5711–5715.
- Lindsay, S.E., Bothast, R.J., Ingram, L.O., 1995. Improved strains of recombinant *Escherichia coli* for ethanol production from sugar mixtures. *Appl. Environ. Microbiol.* 43, 70–75.
- Miller, J.H., 1992. *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Plainview, N.Y..
- Nair, N.U., Zhao, H.M., 2008. Evolution in reverse: engineering a D-xylose-specific xylose reductase. *ChemBioChem* 9, 1213–1215.
- Nichols, N.N., Dien, B.S., Bothast, R.J., 2001. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. *Appl. Microbiol. Biotechnol.* 56, 120–125.
- Panda, A.K., Khan, R.H., Mishra, S., Rao, K.B.C.A., Totey, S.M., 2000. Influences of yeast extract on specific cellular yield of Ovine growth hormone during fed-batch fermentation of *E. coli*. *Bioprocess Biosys. Eng.* 22, 379–383.
- Parajo, J.C., Dominguez, H., Dominguez, J.M., 1998. Biotechnological production of xylitol. Part 1: interest of xylitol and fundamentals of its biosynthesis. *Bioresour. Technol.* 65, 191–201.
- Roberts, M., Riedy, C., Coldwell, S., Nagahama, S., Judge, K., Lam, M., Kaakko, T., Castillo, J., Milgrom, P., 2002. How xylitol-containing products affect cariogenic bacteria. *J. Am. Dent. Assoc.* 133, 435–441.
- Saha, B.C., 2003. Hemicellulose bioconversion. *J. Ind. Microbiol. Biotechnol.* 30, 279–291.
- Silva, S.S., Santos, J.C., Carvalho, W., Aracava, K.K., Vitolo, M., 2003. Use of a fluidized bed reactor operated in semi-continuous mode for xylose-to-xylitol conversion by *Candida guilliermondii* immobilized on porous glass. *Process Biochem.* 38, 903–907.
- Woodyer, R., Simurdiak, M., van der Donk, W.A., Zhao, H.M., 2005. Heterologous expression, purification, and characterization of a highly active xylose reductase from *Neurospora crassa*. *Appl. Environ. Microbiol.* 71, 1642–1647.
- Zhu, Y., Lin, E.C.C., 1988. A mutant *crp* allele that differentially activates the operons of the *fuc* regulon in *Escherichia coli*. *J. Bacteriol.* 170, 2352–2358.