Metabolic Engineering 31 (2015) 112–122

Contents lists available at ScienceDirect

Metabolic Engineering

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

# Efficient production of xylitol from hemicellulosic hydrolysate using engineered *Escherichia coli*

Buli Su [1], Mianbin Wu [*,1], Zhe Zhang, Jianping Lin, Lirong Yang

*Key Laboratory of Biomass Chemical Engineering of Ministry of Education, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China*

ARTICLE INFO

*Article history:*
Received 10 December 2014
Received in revised form
7 July 2015
Accepted 10 July 2015
Available online 18 July 2015

*Keywords:*
Xylitol
Translation initiation rate
mRNA secondary structures
Xylose reductase
Metabolic engineering
Hemicellulosic hydrolysate

ABSTRACT

A metabolically engineered *Escherichia coli* has been constructed for the production of xylitol, one of the top 12 platform chemicals from agricultural sources identified by the US Department of Energy. An optimal plasmid was constructed to express xylose reductase from *Neurospora crassa* with almost no inclusion bodies at relatively high temperature. The phosphoenolpyruvate-dependent glucose phosphotransferase system (*ptsG*) was disrupted to eliminate catabolite repression and allow simultaneous uptake of glucose and xylose. The native pathway for D-xylose catabolism in *E. coli* W3110 was blocked by deleting the xylose isomerase (*xylA*) and xylulose kinase (*xylB*) genes. The putative pathway for xylitol phosphorylation was also blocked by disrupting the phosphoenolpyruvate-dependent fructose phosphotransferase system (*ptsF*). The xylitol producing recombinant *E. coli* allowed production of 172.4 g $L^{-1}$ xylitol after 110 h of fed-batch cultivation with an average productivity of 1.57 g $L^{-1}$ $h^{-1}$. The molar yield of xylitol to glucose reached approximately 2.2 (mol xylitol $mol^{-1}$ glucose). Furthermore, the recombinant strain also produced about 150 g $L^{-1}$ xylitol from hemicellulosic sugars in modified M9 minimal medium and the overall productivity was 1.40 g $L^{-1}$ $h^{-1}$, representing the highest xylitol concentration and productivity reported to date from hemicellulosic sugars using bacteria. Thus, this engineered *E. coli* is a candidate for the development of efficient industrial-scale production of xylitol from hemicellulosic hydrolysate.



## 1. Introduction

Xylitol is a five-carbon sugar alcohol that is as sweet as sucrose but has far fewer dietary calories. In 2004 it was also identified from a list of more than 300 candidates by the US Department of Energy (DOE) as one of the 12 most important building block chemicals that can be produced from biomass (Werpy et al., 2004). The precursor of xylitol, xylose is the second most abundant sugar from lignocellulosic biomass and its utilization is critical for efficient and commercial microbial conversion of biomass-derived substrates into various compounds, such as biofuels and xylitol, so efficient xylose utilization is very important to the xylitol industry (Kim et al., 2012; Latimer et al., 2014; Parachin et al., 2011).

Xylitol is currently manufactured chemically by the reduction of D-xylose derived from hemicellulosic hydrolysates of biomass materials in the presence of Raney nickel catalysts. This chemical process requires expensive separation and purification, because only pure xylose can be reduced chemically (Granstrom et al., 2007). Clearly, this method is not economical or environmentally friendly. Alternative bio-based routes to xylitol production have been developed and considerable efforts has been put into producing xylitol from hemicellulosic hydrolysates (Albuquerque et al., 2014); however, to date there has been no commercially feasible bioprocess developed can compete with chemical reduction in terms of yield and cost.

In recent decades, various other strategies for the biotechnological production of xylitol have been explored (Su et al., 2013); however, few studies have focused on optimizing gene expression, especially at relatively high temperatures. In bacterial expression systems, some genes yield very poor expression levels or cannot be expressed using many commercial expression vectors. This is possibly because of base-pairing between the ribosome-binding site (RBS) and the translation initiation region, which inhibits translation initiation (Mutalik et al., 2013). The translation initiation rate is determined by multiple molecular interactions including hybridization between 16S rRNA and the mRNA at the Shine–Dalgarno (SD) sequence (de Smit and van Duin, 1990), spacing between the SD and start codon and its effects on ribosomal stretching or compression (Chen et al., 1994), and non-specific

* Corresponding author. Fax: +86 571 87953080.
*E-mail addresses:* bolysu@gmail.com (B. Su), wumb@zju.edu.cn (M. Wu), 979987841@qq.com (Z. Zhang), linjp@zju.edu.cn (J. Lin), lryang@zju.edu.cn (L. Yang).
[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.ymben.2015.07.003
1096-7176/

binding between the 30S ribosomal platform domain and the upstream portion of the 5′ untranslated region, where the ribosome can engage in sliding and selective unfolding of structures (Espah Borujeni et al., 2014). Based on this theory, an algorithm was constructed and a software implementation became available for the predictive design of mRNA to control translation efficiency (Salis, 2011). The predictions of this algorithm are reasonably accurate for a diverse range of bacteria including non-model bacterial hosts (Farasat et al., 2014). Thus, this approach can accelerate the construction of genetic systems by rationally optimizing protein expression.

Synonymous mutations in the coding-sequence (Kudla et al., 2009), N-terminal codon bias (Goodman et al., 2013) and different mRNA secondary structures introduced by synonymous mutations in the translation initiation region (Cheong et al., 2015) also affect translation initiation rates and alter protein expression levels. Furthermore, synonymous mutation can influence cotranslational folding and modulate protein function, misfolding, and aggregation (Nissley and O'Brien, 2014). It would thus be a useful means of balancing the inclusion bodies and protein production level for industrial applications.

*E. coli* is an ideal host strain for efficient biotechnological production of various high-value chemical building blocks (Yu et al., 2011), and engineered *E. coli* strains have been developed to produce xylitol from hemicellulosic sugars (Zhao et al., 2011). In many cases recombinant *E. coli* has been shown to produce relatively low yields of many recombinant proteins at high temperatures (usually above 30 °C), because most of the recombinant proteins form inclusion bodies (Schein, 2010). To overcome this obstacle, various strategies have been proposed, including expression at a reduced temperature, reduction of the expression rate, random mutation of the target gene, the use of fusion protein technologies, and co-expression of molecular chaperons (Sorensen and Mortensen, 2005). Cultivation at reduced temperatures is the best known and simplest technique. However, bacterial growth is decreased at low temperatures, resulting in a decreased amount of biomass, and the activities of the enzymes required during fermentation are markedly decreased at these temperatures. Moreover, the consumption of energy required to maintain low temperatures (usually 18 °C) may be an expensive investment. Therefore, low temperature cultivation is not convenient in industrial applications. Here, we explored a strategy to obtain greater levels of soluble protein at relatively high temperatures using synonymous mutations.

In this paper, the construction of a xylitol-producing *E. coli* strain is described (Fig. 1). Various primers with synonymous codons were designed and synthesized to introduce rationally mRNA secondary structures of XR. The ptsG was disrupted to allow simultaneous uptake of glucose and xylose, and to continuously provide sufficient NADPH for the reduction of xylose. The native D-xylose catabolic pathways were blocked by disrupting *xylA* and *xylB* to avoid the consumption of D-xylose for cellular growth. The potential pathway for xylitol phosphorylation was also blocked by disrupting ptsF to reduce the accumulation of xylitol–phosphate. The engineered strain was cultured with glucose added as a carbon source for growth and reduction equivalents, and D-xylose as a substrate for xylitol production.

## 2. Materials and methods

### 2.1. General

The strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used as a host for constructing the recombinant plasmids and *E. coli* W3110 or BL21 (DE3) was used as a host strain. The primer pairs used in this study are listed in Table 5. The nucleotide sequence of xylose reductase (GenBank no.: NCU08384.1) determined by Nair and Zhao (2008) and various oligonucleotide primers were synthesized directly by Sangon Biotech (Shanghai, China). PrimeSTAR Max DNA Polymerase, restriction endonuclease, T4 DNA ligase and their appropriate buffers were purchased from Takara (Dalian, China). Gel DNA Purification Kits and Plasmid Midi Kits were obtained from AxyPrep (Hangzhou, China). Isopropyl β-D-1-thiogalactopyranoside (IPTG), L-arabinose, 4-Morpholinopropanesulfonic acid (MOPS), ampicillin, NADPH and kanamycin were purchased from GEN-VIEW (GEN-VIEW SCIENTIFIC INC., US). Tryptone and yeast extraction were from Oxoid (Basingstoke, UK). All other compounds were of reagent grade or higher quality.

### 2.2. Genetic methods

All recombinant plasmids were constructed based on plasmid vector pET-30a(+) and pTrc99a. Targeted gene deletions were performed using the λRed/ET system (Datsenko and Wanner, 2000). Plasmid pKD46 was used as the Red recombinase expression vector, pKD3 was used as the template plasmid for PCR amplification of disruption cassettes containing the chloramphenicol resistance gene, and pCP20 was used as the resistance marker eliminating plasmid. For *ptsG* gene deletion, PCR products were amplified with the primers Del-ptsG-P1 and Del-ptsG-P2, using pKD3 as a template. The PCR products contained the cat gene flanked by FRT (Flp recognition target) and 50 nt of sequence identical to the target locus were transformed into cells expressing λRed recombinase proteins (encoded on pKD46). Gene replacement was selected for on chloramphenicol plates and verified by functional assay and PCR. The resistance marker was then removed by thermo-inducible Flp recombinases 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 *xylAB* and *ptsF* were ;performed via the same method, using appropriate primers (Del-xylA-P1, Del-xylA-P2, xylAB-FRT-P1, xylAB-FRT-P2, Del-xylB-P1and Del-xylB-P2 for *xylAB*; Del-ptsF-up-P1, Del-ptsF-up-P2, ptsF-FRT-P1, ptsF-FRT-P2, Del-ptsF-do-P1 and Del-ptsF-do-P2 for *ptsF*) but with about 800 nt of sequence identical to the target locus. All deletions were verified by PCR using appropriate primers (Table 5); ptsG-check1 and ptsG-check2 for *ptsG*; Del-xylA-P1 and Del-xylB-P2 for *xylAB*; Del-ptsF-up-P1 and Del-ptsF-do-P2 for *ptsF*.

*E. coli* strains harboring recombinant plasmids were cultured in Luria-Bertani (LB) medium (5 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ tryptone, and 10 g $L^{-1}$ NaCl) supplemented with 100 μg $mL^{-1}$ ampicillin, 34 μg $mL^{-1}$ chloramphenicol or 50 μg $mL^{-1}$ kanamycin at 37 °C, 200 rpm. For induction, a subculture grown overnight was inoculated into fresh medium to a final concentration of 2%. Cell culture optical density was measured spectrophotometrically at 600 nm ($OD_{600}$). When the $OD_{600}$ reached 0.6–1.0, 0.1 mM IPTG was added, and the cells were cultured for a further 15 h at 18 °C or 30 °C, 200 rpm.

### 2.3. RNA secondary structure predictions and plasmid construction

To evaluate the effects of mRNA secondary structures around the translation initiation region on gene expression, various primers with synonymous codons that allowed for the introduction of rationally designed mRNA secondary structures of XR were designed and synthesized. The secondary structures were predicted based on a free energy model that calculated the total change in Gibbs free energy upon binding of the 30S ribosome complex to a ribosome-binding site on the mRNA and the translation initiation rates were evaluated using the RBS Calculator v1.1 available online (Salis et al., 2009) (https://salis.psu.edu/soft