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Enhanced xylitol production through simultaneous co-utilization of cellobiose and xylose by engineered *Saccharomyces cerevisiae*

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ABSTRACT

As *Saccharomyces cerevisiae* cannot utilize xylose as a carbon source, expression of *XYL1* coding for xylose reductase (XR) from *Scheffersomyces* (*Pichia*) *stipitis* enabled production of xylitol from xylose with a high yield. However, insufficient supply of NAD(P)H for XR and inhibition of xylose uptake by glucose are identified as major constraints for achieving high xylitol productivity. To overcome these problems, we engineered *S. cerevisiae* capable of converting xylose into xylitol through simultaneous utilization of xylose and cellobiose. An engineered *S. cerevisiae* (D-10-BT) expressing XR, cellodextrin transporter (*cdt-1*) and intracellular β-glucosidase (*gh1-1*) produced xylitol via simultaneous utilization of cellobiose and xylose. The D-10-BT strain exhibited 40% higher volumetric xylitol productivity with co-consumption of cellobiose and xylose compared to sequential utilization of glucose and xylose. Furthermore, the overexpression of *S. cerevisiae ALD6*, *IDP2*, or *S. stipitis ZWF1* coding for cytosolic $NADP^+$-dependent dehydrogenases increased the intracellular NADPH availability of the D-10-BT strain, which resulted in a 37–63% improvement in xylitol productivity when cellobiose and xylose were co-consumed. These results suggest that co-utilization of cellobiose and xylose can lead to improved xylitol production through enhanced xylose uptake and efficient cofactor regeneration.

1. Introduction

Xylitol is a five-carbon sugar alcohol which is similar in sweetness to sucrose (Hyvonen et al., 1982). Xylitol is widely used as a sugar substitute in various food products, such as chewing gum and candy, because it inhibits dental caries and is also low in calories (Makinen, 1992). In addition, xylitol is one of the high-value bio-based chemicals that can be produced from cellulosic sugars. Xylitol can be used as a building block for various chemical compounds such as xylaric acid or glycols (Werpy et al., 2004). Xylitol is currently produced by a chemical hydrogenation process (Wisniak et al., 1974). However, xylitol yields from the chemical conversion process are lower than those from biological processes because chemical reduction produces byproducts (Melaja and Hamalainen, 1977). Therefore, microbial conversion of xylose to xylitol is being studied to replace the chemical process using metabolically engineered yeast strains and bacteria (Hallborn et al., 1991; Nigam and Singh, 1995; Akinterinwa and Cirino, 2009; Jeon et al., 2012).

The natural xylose-fermenting yeast, *Candida* sp. can convert xylose into xylitol, however there are potential drawbacks for using this yeast for xylitol production. *Candida* sp. can use xylose as a carbon source for cell growth and metabolism. This xylose assimilating capability often results in lower xylitol yields (Chung et al., 2002). Some *Candida* sp. exhibit a human pathogenic nature under opportunistic situations so microbial production of xylitol by *Candida* sp. might not be desirable for large-scale fermentations (Granstrom et al., 2007). In this context, xylitol production by *Saccharomyces cerevisiae* may be advantageous because *S. cerevisiae* is generally recognized as safe (GRAS). Although *S. cerevisiae* cannot ferment xylose, it can be engineered to produce xylitol as genetic manipulations of this yeast are easier than any other eukaryotes (Romanos et al., 1992).

Introduction of *XYL1*, coding for xylose reductase (XR) from *Scheffersomyces* (*Pichia*) *stipitis*, is necessary for *S. cerevisiae* to metabolize xylose and produce xylitol. Engineered *S. cerevisiae* expressing *XYL1* can produce xylitol with a theoretical yield of 1.00 g/g because the engineered strain cannot assimilate xylose as

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a carbon source. However, supply of co-substrates enabling cell growth and metabolism is required for xylitol production in the engineered *S. cerevisiae* (Hallborn et al., 1991). When glucose is used as a co-substrate, efficient xylitol production is hindered as glucose inhibits xylose transport severely (Hallborn et al., 1994; Meinander and Hahn-Hägerdal, 1997). It was reported that transportation of xylose in *S. cerevisiae* is highly inhibited by glucose as xylose transport in this yeast is mediated by hexose transporters. Therefore, xylose can be converted into xylitol only after glucose is depleted when a mixture of glucose and xylose is used (Trumbly, 1992). In order to alleviate glucose repression on xylose transport, glucose-limited fed-batch fermentations can be performed where glucose concentrations are maintained at lower levels for allowing transportation of xylose into cells (Lee et al., 2000). However, controlling glucose concentrations at desired levels in a large-scale fermentation is not only difficult, but also might result in insufficient supply of NAD(P)H that is used by XR as a cofactor. Although other carbon sources such as ethanol or glycerol can be used as a co-substrate without inhibiting xylose transport (Van Vleet et al., 2008), cofactor regeneration capacities of ethanol or glycerol under oxygen-limited conditions are not as good as glucose. Because of these two major problems (glucose repression and insufficient cofactor regeneration), engineered *S. cerevisiae* expressing XR suffers from lower volumetric productivities, whereas it showed much higher xylitol yields than *Candida* sp.

In order to bypass these problems, we demonstrate a co-utilization strategy whereby transportation of xylose is not inhibited while NAD(P)H is efficiently generated through simultaneous cofermentation of cellobiose (a dimer of glucose) and xylose which are prevalent in cellulosic hydrolyzates. As native *S. cerevisiae* cannot utilize cellobiose, we introduced a cellodextrin transporter (*cdt-1*) and intracellular β-glucosidase (*gh1-1*) from the cellulolytic fungi, *Neurospora crassa* (Galazka et al., 2010; Ha et al., 2011), into the engineered *S. cerevisiae* which has integrated *XYL1* from *S. stipitis*. The resulting engineered *S. cerevisiae* (D-10-BT) not only produced xylitol constitutively without glucose repression, but also showed higher xylitol production rates when cellobiose and xylose are co-consumed as compared to when glucose and xylose are sequentially utilized. We also observed that overexpression of cytosolic $NADP^+$- dependent dehydrogenases, which are the major sources of NADPH in *S. cerevisiae* (Minard and McAlister-Henn, 2005), resulted in further improvement of xylitol productivity in the D-10-BT strain during co-utilization of cellobiose and xylose.

2. Materials and methods

2.1. Strain and plasmid construction

Strains and plasmids used in this study are described in Table 1. *S. cerevisiae* D452-2 (*MATalpha, leu2, his3, ura3, and can1*) was used for engineering of cellobiose metabolism as well as *XYL1* integration. *Escherichia coli* DH5 (*F– recA1 endA1 hsdR17 [rK– mK+] supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation. pYS10 (Jin and Jeffries, 2003), containing *S. stipitis XYL1* under the control of *S. cerevisiae TDH3* promoter, was linearized by *Hpa*I and genome-integrated into D452-2 to yield the D-10 strain. For expression of β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*) from *N. crassa* (Galazka et al., 2010; Ha et al., 2011), two open reading frames (*cdt-1* and *gh1-1*) were placed between the *PGK1* promoter and *CYC1* terminator. Each expression cassette of β-glucosidase and cellodextrin transporter was amplified by PCR with primers T7-SwaI (GGCATTTAAATTAATACGACTCACTATAGGG) and T3-SwaI (GGCATTTAAATAATTAACCCTCACTAAAGGG) which have *Swa*I blunt enzyme site (underlined). After *Swa*I treatment, two cassettes were cloned into *Eco*RV treated pRS425 vector to generate pRS425-BT. To overexpress $NADP^+$-dependent dehydrogenases, the *ALD6* (*S. cerevisiae* aldehyde dehydrogenase), *IDP2* (*S. cerevisiae* isocitrate dehydrogenase), and *SsZWF1* (*S. stipitis* glucose-6-phosphate dehydrogenase) genes were cloned separately into the pRS42KTEF plasmid containing the KanMX marker, *TEF* promoter, and *CYC1* terminator. The resulting plasmids (pRS42KTEF-ALD6, pRS42KTEF-IDP2, and pRS42KTEF-SsZWF1) were individually transformed into the *S. cerevisiae* D-10-BT strain, and the transformants (D-10-BT-C, D-10-BT-ALD6, D-10-BT-IDP2, and D-10-BT-SsZWF1, respectively) were selected on a YP agar plate (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L agar) containing 20 g/L of cellobiose and 200 μg/mL of G418.

2.2. Media and culture conditions

E. coli was grown in Luria-Bertani medium; 50 μg/mL of ampicillin was added to the medium when required. Yeast strains

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
S. cerevisiae D452-2	*MATalpha, leu2, his3, ura3, and can1*	Hosaka et al. (1992)
S. cerevisiae D-BT	*S. cerevisiae* D452-2 (pRS425-BT)	Ha et al. (2011)
S. cerevisiae D-10	Isogenic of D452-2 except for *leu2*::*LEU2-SsXYL1*	This study
S. cerevisiae D-10-BT	*S. cerevisiae* D-10 (pRS425-BT)	This study
S. cerevisiae D-10-BT-C	*S. cerevisiae* D-10-BT (pRS42KTEF)	This study
S. cerevisiae D-10-BT-ALD6	*S. cerevisiae* D-10-BT (pRS42KTEF-ALD6)	This study
S. cerevisiae D-10-BT-IDP2	*S. cerevisiae* D-10-BT (pRS42KTEF-IDP2)	This study
S. cerevisiae D-10-BT-SsZWF1	*S. cerevisiae* D-10-BT (pRS42KTEF-SsZWF1)	This study
Plasmids		
pYS10	pRS305 $TDH3_P$-*SsXYL1*-$TDH3_T$	Jin and Jeffries (2003)
pRS425	*LEU2*, 2-μm origin	Christianson et al. (1992)
pRS425-BT	*gh1-1* and *cdt-1* under the control of PGK1 promoter in pRS425	Ha et al. (2011)
pRS42K	2-μm origin, drug resistance marker ($G418^R$)	Taxis and Knop (2006)
pRS42KTEF	2-μm origin, drug resistance marker ($G418^R$), TEF_P and $CYC1_T$	This study
pRS42KTEF-ALD6	*ALD6* under the control of TEF promoter in pRS42K	This study
pRS42KTEF-IDP2	*IDP2* under the control of TEF promoter in pRS42K	This study
pRS42KTEF-SsZWF1	*SsZWF1* under the control of TEF promoter in pRS42K	This study