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Effects of Xylose Reductase Activity on Xylitol Production in Two-Substrate Fermentation of Recombinant *Saccharomyces cerevisiae*

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Abstract Three recombinant *Saccharomyces cerevisiae* strains showing different levels of xylose reductase activity were constructed to investigate the effects of xylose reductase activity and glucose feed rate on xylitol production. Conversion of xylose to xylitol is catalyzed by xylose reductase of *Pichia stipitis* with cofactor NAD(P)H. A two-substrate fermentation strategy has been employed where glucose is used as an energy source for NADPH regeneration and xylose as substrate for xylitol production. All recombinant *S. cerevisiae* strains yielded similar specific xylitol productivity, indicating that xylitol production in the recombinant *S. cerevisiae* was more profoundly affected by the glucose supply and concomitant regeneration of cofactor than the xylose reductase activity itself. It was confirmed in a continuous culture that the elevation of the glucose feeding level in the xylose-conversion period enhanced the xylitol productivity in the recombinant *S. cerevisiae*.

Key words: *Saccharomyces cerevisiae*, glucose, xylitol, xylose reductase, NAD(P)H

Xylitol is a naturally-occurring five-carbon sugar alcohol [3]. Its high sweetening power, anti-cariogenic properties, and possibilities for use in diabetic food products make xylitol an attractive substitute for sucrose in a wide range of foods and beverages [19]. Most research efforts on the biological production of xylitol have been made with yeasts, especially species of genus *Candida* [2, 3, 9, 16, 18]. With xylose-fermenting yeasts found in nature, high xylitol yields may be difficult to obtain, since a certain fraction of xylose must be used for cell growth and endogenous metabolism. Recombinant *S. cerevisiae* strains transformed with the xylose reductase gene (*XYL1*) of *Pichia stipitis* and *Candida shehatae* allowed the efficient conversion of xylose to xylitol with approximate theoretical yield [4, 6, 7, 13, 25]. Cofactors NADH and NADPH are essential for the enzymatic reduction of xylose by xylose reductase (XR) [12, 23]. Accordingly, xylitol production by the recombinant *S. cerevisiae* requires the simultaneous metabolism of cosubstrates such as glucose and ethanol for continuous generation of NAD(P)H and maintenance energy [13–15].

In this study, the effects of xylose reductase activity and glucose feed rate on the xylitol production in the recombinant *S. cerevisiae* were studied in a series of glucose-limited fed-batch and continuous cultures. Glucose feeding rates were controlled to keep glucose concentrations in the medium low enough to avoid glucose-mediated inhibition of xylose transport into the yeast cells.

Materials and Methods

Strains and Plasmids

Escherichia coli DH5α was used as a host for plasmid preparation. The xylose reductase gene of *P. stipitis* was expressed in *S. cerevisiae* BJ3505 (ATCC 208281, MAT. α, *trp1*, *ura3*). Plasmids pUXR and pY2XR [4, 13] were kindly donated by Professor Y. D. Choi at Seoul National University, and pYADE4 was a generous gift from Professor Martin L. Pall at Washington State University (Pullman, WA, U.S.A.). Plasmids pYADHXR and YIpXR were constructed in this study. The GAPDH promoter in pY2XR plasmid was replaced with the ADH2 promoter which is derepressed to allow the expression of the xylose reductase gene to take place under low glucose environment [20]. Plasmid pUXR was further digested with *Bsp*EI, and the sticky ends were end-filled with the Klenow fragment.

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This linearized DNA was digested again with *Kpn*I to isolate the xylose reductase gene fragment. Plasmid pYADHXR (7.1 kb, URA3) was constructed by introducing this fragment between *Sma*I and *Kpn*I sites of pYADE4 vector [1] and then transformed into *S. cerevisiae* BJ3505 strain. YIpXR plasmid (7.6 kb, URA3) was constructed by inserting the structural gene of xylose reductase along with the GAPDH promoter/terminator fragment into YIp5 vector [24], linearized with *Apa*I restriction enzyme and then transformed into *S. cerevisiae* BJ3505.

DNA Manipulation and Yeast Transformation

All DNA manipulations were carried out based on the methods described by Sambrook *et al.* [22]. Restriction enzymes purchased from New England Biolabs (Beverly, MA, U.S.A.) were used under the reaction conditions recommended by the supplier. Transformation of *S. cerevisiae* was carried out by using the yeast transformation kit (QBiogene, Carlsbad, CA, U.S.A.) following the manufacturer's instructions.

Media and Culture Conditions

LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) was used to cultivate *E. coli*, and YPD medium (20 g bacto-peptone, 10 g yeast extract, 20 g glucose per liter) was used to cultivate the host *S. cerevisiae* strain. Yeast synthetic drop-out supplements (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added to YNB medium (6.7 g yeast nitrogen base w/o amino acid, 20 g glucose per liter) to prepare YNB drop-out medium that was required for making a selection of yeast transformants. A synthetic medium [13, 17], supplemented with casein hydrolysate (5 g/l), $(NH_4)_2SO_4$ (10 g/l), and KH_2PO_4 (10 g/l), was used for the fed-batch cultivation of recombinant *S. cerevisiae* strains. Medium compositions are summarized in Table 1.

Table 1. Medium composition for fed-batch fermentations of recombinant *Saccharomyces cerevisiae* strains harboring the *XYL1* gene of *Pichia stipitis*.

Components	Concentration (g/l)
Xylose	20
Glucose	20
$(NH_4)_2SO_4$	5.0
KH_2PO_4	2.85
$CaCl_2 \cdot 2H_2O$	0.08
$MgSO_4 \cdot 7H_2O$	0.60
Trisodium citrate dihydrate	0.03
Trace metal and vitamin solution*	

*Trace metal and vitamin solution contains 50 mg $FeCl_3 \cdot 6H_2O$, 15 mg $ZnSO_4 \cdot 7H_2O$, 0.4 mg $CuSO_4 \cdot 5H_2O$, 7.5 mg H_3BO_3, 1 mg KI, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 2.8 mg $CoCl_2 \cdot 6H_2O$, 16 mg $MnSO_4 \cdot H_2O$, 0.05 mg D-biotin, 80 mg myo-inositol, 5 mg pyridoxine·HCl, 7.5 mg nicotinic acid, and 2 mg thiamine·HCl per liter.

A loopful of recombinant *S. cerevisiae* was transferred to a test tube containing 5 ml of YNB medium and then incubated overnight at 30°C. Shake flask seed cultures were carried out in 500 ml of baffled flasks containing 100 ml of YNB medium (6.7 g yeast nitrogen base without amino acid, 1.92 g of yeast synthetic drop-out supplements, and 20 g of glucose per liter). For two-substrate fed-batch fermentations, cells were grown in a batch mode until initially added glucose was completely consumed, and the cultivation mode was then switched to fed-batch by feeding sugar solutions. A mixture of glucose and xylose at 800 g/l was used in the fed-batch phase, and their residual concentrations in the fermenter were manipulated by using different feeding strategies. Continuous fermentations were performed in YNB medium supplemented with 10 g/l of glucose, 30 g/l of xylose, and 1.92 g/l of yeast synthetic drop-out supplement. An agitation speed was set at 400 rpm and aeration rate was 1 vvm. Medium pH was automatically controlled at 5.0 by adding 2 N NaOH or 2 N HCl. Temperature was maintained at 30°C throughout the cultivation.

Analysis

Optical density was converted into dry cell mass concentration by using the following equation.

$$\text{Dry cell mass (g/l)} = 0.17 \times OD_{600}$$

After centrifugation, samples were stored at 4°C until further analysis. For xylose reductase activity assay, a culture volume equivalent to 1×10^8 cells was collected by centrifugation and stored at -80°C. Concentrations of xylose, glucose, and xylitol were analyzed by HPLC (Knauer, Berlin, Germany) equipped with a Carbohydrate Analysis column (Waters, Milford, MA, U.S.A.). Ethanol was analyzed by a gas chromatograph (YongLin Co., Seoul, Korea) equipped with a flame ionization detector. The flow rate of the carrier gas, nitrogen, was kept constant at 30 ml/min. The injector and detector were maintained at 200°C, and the column (Carbowax 20M, Hewlett-Packard, Palo Alto, CA, U.S.A.) at 150°C. Glucose concentrations were also determined with an enzymatic kit (Yeongdong Pharm., Seoul, Korea), and a D-glucose analyzer (YSI Life Sciences Inc., Yellow Springs, OH, U.S.A.) was used for fed-batch and continuous fermentations.

Xylose Reductase Activity Assay

Xylose reductase activity was determined as described by Webb and Lee [26] with slight modifications. Cells (1×10^8) were resuspended in 1 ml of disruption buffer [20 mM of Tris·Cl (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 1 mM of PMSF]. One gram of glass beads was added to the suspension and then the mixture was homogenized with a bead beater at 5,000 rpm for 20 sec. This was repeated six

times, followed by centrifugation at 8,000 rpm and 4°C to remove cell debris. The supernatant was used for enzyme assay. The standard assay volume of 200 µl contained 50 mM potassium phosphate buffer (pH 6.0), 0.2 mM NADPH, and 13.3 mM of D-xylose. One unit (U) of xylose reductase (XR) activity was defined as the amount of enzyme to oxidize 1 µmole NADPH per min at 30°C. Specific activity was obtained by dividing the enzyme activity by protein concentration or dry cell mass concentration. Protein concentrations were determined by using a micro protein determination kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) with human serum albumin as a standard.

RESULTS AND DISCUSSION

Effect of Xylose Reductase Activity

In glucose-limited fed-batch cultures of the recombinant *S. cerevisiae* strain harboring pY2XR plasmid [3, 13], specific xylitol productivity was drastically reduced along with a decrease in specific xylose reductase activity in the fed-batch phase. Hence, the relationship between xylose reductase activity and xylitol productivity was investigated by constructing recombinant *S. cerevisiae* strains having different levels of xylose reductase activity. The ADH2 promoter, whose activity is derepressed when glucose is depleted, was employed to allow the expression of xylose reductase under a low glucose environment.

Glucose-limited fed-batch cultures were performed with BJ3505/pYADHXR and BJ3505/YIpXR strains through monitoring ethanol concentrations, as we described previously [3], and the results were compared with that of BJ3505/pY2XR. All three recombinant strains showed similar fermentation patterns and profiles of BJ3505/pYADHXR strain as depicted in Fig. 1. The specific activity of xylose reductase and xylitol productivity of the three recombinant strains are summarized in Table 2. As shown in Fig. 1, the specific xylitol productivity of the BJ3505/pYADHXR strain was significantly diminished and remained at a basal level even with continuous feeding of glucose in the fed-batch phase. Accordingly, it was considered that the glucose feeding strategy caused glucose starving conditions and hence the specific xylitol productivity decreased in the fed-batch phase even with a relatively constant level of xylose reductase activity throughout the cultivation.

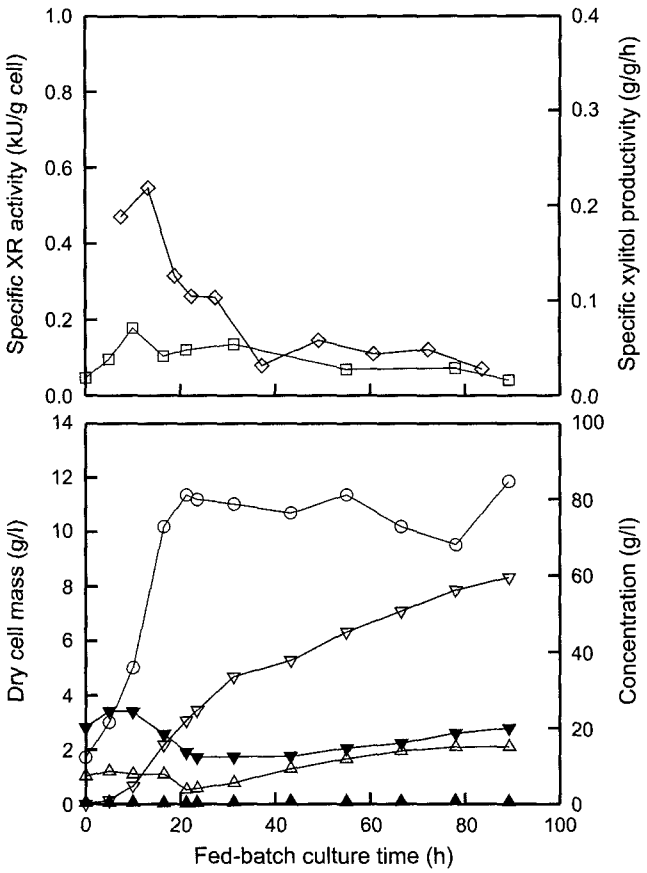

Fig. 1. Fed-batch fermentation profiles of *S. cerevisiae* BJ3505/pYADHXR grown in a synthetic medium at 30°C at pH 5.0.
Cells were grown overnight with initial concentrations of 20 g/l glucose and 20 g/l xylose in a 2.5 l fermenter, and fed-batch mode was initiated after the depletion of initially added glucose. A mixture of glucose and xylose at 800 g/l was fed to manipulate their residual concentrations in the fermenter. ○: dry cell mass; ▲: glucose; ▼: xylose; △: ethanol; ▽: xylitol; □: specific xylose reductase activity; ◇: specific xylitol productivity.

Table 2 shows that the specific xylose reductase activity of the BJ3505/pY2XR strain was maintained significantly higher than those of the BJ3505/pYADHXR and BJ3505/YIpXR strains. However, the specific activity of xylose

Table 2. Xylose reductase activities and specific xylitol productivities for the three *S. cerevisiae* BJ3505 strains in the two-substrate fed-batch cultures.

	Recombinant *S. cerevisiae* BJ3505 strains								
	BJ3505/pY2XR			BJ3505/pYADHXR			BJ3505/YIpXR		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
Specific xylose reductase activity (kU/g cell)	1.81	0.96	0.74	0.21	0.32	0.27	0.14	0.15	0.11
Specific xylitol productivity (g/g/h)	0.27	0.19	0.13	0.19	0.22	0.13	0.10	0.12	0.05

#1, #2, and #3 represent the first three sampling terms in the fed-batch cultures.

reductase of BJ3505/pY2XR decreased drastically, as observed in our previous study [4], while those of pYADHXR and YIpXR strains remained relatively stable.

Similar experimental results were reported by Meinander and Hahn-Hägerdal [15] in which the expression level of xylose reductase with the ADH2 promoter was enhanced by twenty folds by using the strong PGK promoter. However, the specific xylitol productivity by the high XR activity strain was only two times higher than that by the low XR activity strain under the limited supply of glucose. On the other hand, enhanced xylitol productivity was obtained by elevating the levels of both xylose reductase activity and cofactor supply through genetic manipulation and increased glucose feeding [4]. Therefore, it appeared that the level of xylose reductase activity alone was not the only important factor in xylitol production. In other words, a decrease of specific xylitol productivity was not caused entirely by low xylose reductase activity, indicating the importance of energy supply and cofactor regeneration for the xylitol production in the recombinant *S. cerevisiae*.

Effect of Glucose Feeding on Xylitol Production

In our previous studies [4, 13], glucose feed rate was modulated to prevent excessive accumulation of ethanol which negatively affects xylitol production [13]. However, insufficient glucose feeding and a lack of concomitant cofactor regeneration are considered to result in a decreased xylitol productivity and also to counterbalance the effects of an elevated xylose reductase level on the xylitol production in the recombinant *S. cerevisiae*.

Accordingly, fed-batch fermentations of the BJ3505/pYADHXR strain were carried out by using different glucose feeding strategies to explore the extent of xylitol productivity dependency on glucose concentration in the medium. Glucose feeding began at 2 ml/h (1.6 g/h) and gradually increased up to 8.3 ml/h (6.6 g/h). Maximum dry cell mass concentration increased up to 20.5 g/l, and overall volumetric xylitol productivity for xylitol was 1.23 g/l·h, corresponding to a 1.8-fold increase in an overall xylitol productivity compared with that shown in Fig. 1. Fed-batch fermentation was carried out further at a higher glucose feeding rate. Glucose feeding was initiated at a rate of 2 ml/h, and gradually increased up to 10.7 ml/h (8.6 g/h) in a shorter period than in the previous fed-batch culture. The maximum dry cell mass concentration of 20.5 g/l and overall volumetric xylitol productivity of 1.62 g/l·h were obtained (Fig. 2). While higher glucose feeding was not effective for increasing final dry cell mass concentration, the xylitol productivity was significantly improved by increasing the glucose supply for xylose conversion in the fed-batch phase. The above result of fed-batch cultivations suggested that a glucose feeding strategy played a very important role in enhancing the xylitol productivity in the recombinant *S. cerevisiae*.

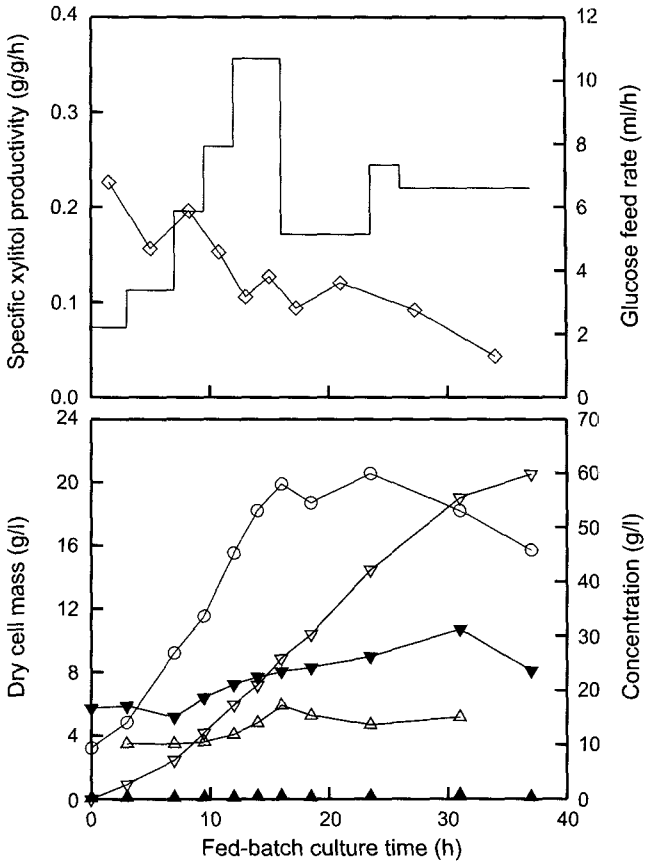

Fig. 2. Fed-batch fermentation profiles of *S. cerevisiae* BJ3505/pYADHXR grown in a synthetic medium at 30°C and pH 5.0.
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A relationship between xylitol productivity and glucose feed rate was further investigated in a continuous cultivation of the BJ3505/pY2XR strain at various dilution rates, ranging from 0.03 to 0.28 1/h (Fig. 3). In the continuous culture of *S. cerevisiae*, the specific glucose consumption rate at a dilution rate of 0.28 1/h was 1.62 g/g cell·h, which was twenty-fold higher than that obtained at a dilution rate of 0.03 1/h. The GAPDH promoter is known to be constitutive, but xylose reductase activity was not detected at low dilution rates below 0.07 1/h, and xylitol production started only at dilution rates higher than 0.21 1/h. Accordingly, the GAPDH-driven expression of xylose reductase was found to be greatly influenced by the glucose consumption rate of the host cell. While specific xylose reductase activity increased more than two folds by increasing dilution rates of 0.21 to 0.28 1/h, there was only 30% increase in specific xylitol productivity, indicating that the xylose reductase activity in the recombinant *S.*

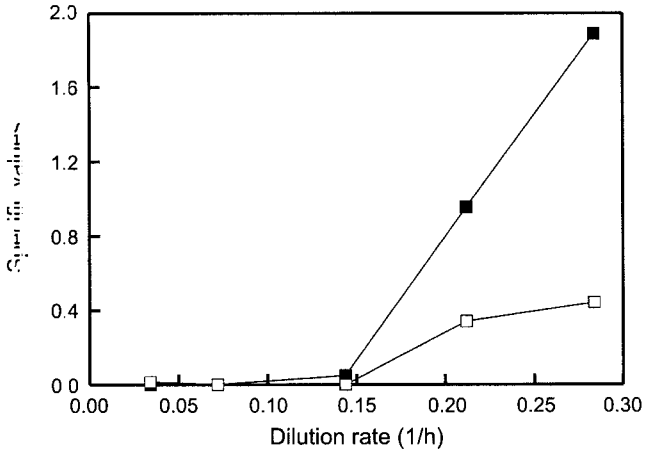

Fig. 3. Continuous fermentation of *S. cerevisiae* BJ3505/pYX2XR.

Concentrations of glucose and xylose in the feeding solution were 10 g/l and 30 g/l, respectively. ■: Specific xylose reductase activity (kU/g cell); □: specific xylitol productivity (g/g/h).

cerevisiae did not correlate with xylitol productivity. Glucose consumption rate has been thought to be an important factor not only for the expression of xylose reductase, but also for the cofactor regeneration, and hence xylitol production in *S. cerevisiae*. Since xylose transport is competitively inhibited by glucose at high dilution rates, it has been suggested that there must be a reasonable tradeoff between the glucose feeding and xylose transport to sufficiently supply cofactors and xylose, and finally, to maximize xylitol productivity.

In *S. cerevisiae*, NADH is abundantly produced by alcohol dehydrogenase I (ADH1) which catalyzes ethanol production from acetaldehyde [21], and xylose reductase from *P. stipitis* prefers NADPH to NADH as a cofactor [2,6]. Therefore, most of NADH that is generated in glycolysis may be directed to the ethanol formation, therefore, NADH can not be utilized evenly for the XR-mediated xylitol production. This hypothesis is supported by the fact that the specific ethanol efflux is not affected by the consumption of NAD(P)H by xylose reductase [15]. It seems that most of xylitol is produced with NADPH generated in the oxidative pentose phosphate pathway (PPP). One of the metabolic roles of PPP is to supply metabolic intermediates for the cellular building blocks. When yeast cells are grown in the medium enriched with yeast extract, the glucose flux to the PPP decreases, resulting in a decrease in NAD(P)H production [5]. In a fed-batch fermentation with YPD medium, specific xylitol productivity of the recombinant *S. cerevisiae* B3505/YIpXR was reduced, compared with the fermentations with the synthetic medium (data not shown). Such a limited flux to the PPP might occur in the glucose-limited fed-batch culture to cause insufficient generation of NAD(P)H, consequently decreasing the xylose reduction rate in *S. cerevisiae*. Lower productivity of xylitol fermentations performed under an anaerobic condition might be accounted for in the same manner [15].

In conclusion, optimization of a glucose feeding strategy is an important prerequisite for efficient regeneration of NAD(P)H, which is required for the enhancement of xylitol production in the recombinant *S. cerevisiae* harboring the xylose reductase gene.

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