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Conversion of Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae* Containing Genes for Xylose Reductase and Xylitol Dehydrogenase from *Pichia stipitis*

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Abstract A recombinant *Saccharomyces cerevisiae*, transformed with the genes encoding xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) originated from *Pichia stipitis* CBS 5776, was developed to directly convert xylose to ethanol. A fed-batch fermentation with the recombinant yeast produced 8.7 g ethanol/l with a yield of 0.13 g ethanol/g xylose consumed.

Key words: Xylose, ethanol, xylose reductase, xylose dehydrogenase, *Saccharomyces cerevisiae*

The potential for producing ethanol from the biomass is innumerous, as 3×10^{21} J of biomass is annually produced by photosynthesis. Cellulosic materials such as sugar cane and maize consist of 40% cellulose, 30% hemicellulose, and 20% lignin on average. Cellulose hydrolyzates are effectively converted to ethanol by a recombinant *S. cerevisiae* [5, 11]. D-Xylose is a major component of the hydrolyzates of hemicellulose from the biomass. Some bacteria, yeasts, and fungi can ferment xylose to produce ethanol, however, they have many disadvantages such as a low ethanol tolerance and by-product formation such as acetic acid. In order to assimilate xylose to a cellular metabolic pathway, yeasts convert xylose to xylulose in a two-step reaction with oxidoreductases. Xylose is first reduced to xylitol by NADPH/NADH-linked xylose reductase (XR), and then xylitol is either excreted from the cell or oxidized to xylulose by NADP/NAD-linked xylitol dehydrogenase (XDH). In contrast, in bacteria, xylose is isomerized to xylulose by xylose isomerase. However, the subsequent metabolic pathways for xylulose are known to be identical in both yeasts and bacteria [3, 4, 7]. *S. cerevisiae* has been used as a strong ethanol producer from hexoses, yet it cannot utilize xylose, as it does not possess a metabolic activity to convert xylose to xylulose. However, it can slowly metabolize xylulose to produce ethanol. Research efforts have been made to metabolically engineer *S. cerevisiae* by introducing into it genes involved in the xylose metabolism of xylose-fermenting yeasts. Köter and Ciriacy [1993] characterized the fermentation properties of recombinant *S. cerevisiae* containing the *XYL1* and *XYL2* genes from *P. stipitis* CBS 5774 and reported that 3 g ethanol/l was produced with an ethanol yield of 0.14 g ethanol/g xylose consumed [8]. Tantirungkly *et al.* [1994] also performed a fed-batch fermentation with a recombinant *S. cerevisiae* strain. They actually improved the ethanol yield of 0.17 g ethanol/g xylose by 210% when compared with simple batch fermentation at the expense of a low ethanol productivity of 0.068 g ethanol/l $\cdot$ h^{-1} [12].

This study was undertaken to establish the xylose utilization and ethanol production patterns of recombinant *S. cerevisiae* containing the genes encoding xylose reductase and xylitol dehydrogenase from *P. stipitis* CBS 5776.

S. cerevisiae 2805 (*Mat α pep::HIS3 prb1-Δ can1 GAL2 his3Δ ura3-52*) was used as the host for transformation. The plasmids used for cloning the *XYL1* and *XYL2* genes were pYPR2831 and YIp5, respectively. The DNA fragment containing the *XYL1* gene between the glyceraldehyde-3-phosphate dehydrogenase promoter and terminator was separated by *Hin*dIII digestion from plasmid pY2XR that had been previously constructed and inserted into the unique YIp5 *Hin*dIII site. The constructed plasmid, YIpXR, was linearized by *Apa*I digestion and used for the integration of *XYL1* into the *S. cerevisiae* chromosome (Fig. 1). Plasmid pY2XDH (Fig. 2) containing the *XYL2* gene between the glyceraldehyde-3-phosphate dehydrogenase promoter and terminator in the episomal yeast expression vector, pYPR2831,

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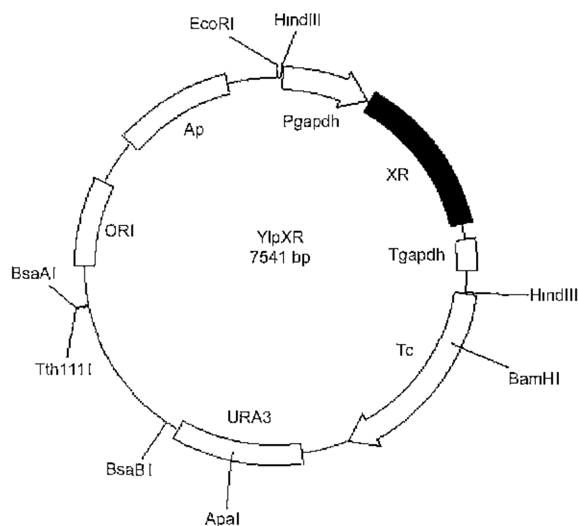

Fig. 1. Genetic map of plasmid YIpXR containing the xylose reductase gene.

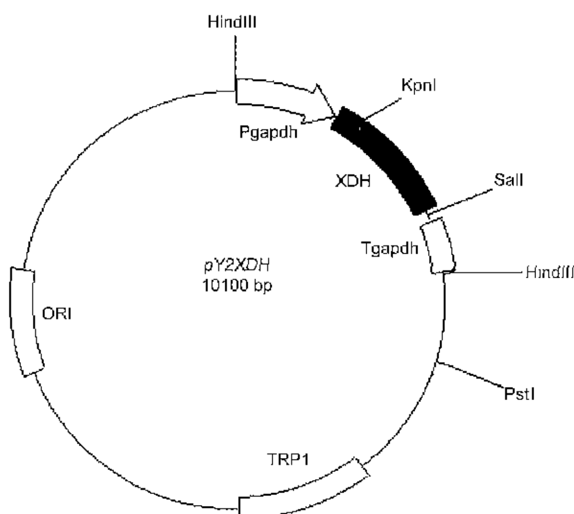

Fig. 2. Genetic map of plasmid pY2XDH containing the xylitol dehydrogenase gene.

was also introduced into the *XYL1*-integrated recombinant *S. cerevisiae* 2805 YIpXR. Yeast transformations were carried out using the Alkali-Cation yeast transformation kit (BIO101, U.S.A.) based on the instruction of the manufacturer. The resulting recombinant was named *S. cerevisiae* 2805 YIpXR:pY2XDH.

Fed-batch fermentation was performed in a 2.5-l fermentor (Korea Fermentor Co., Korea) in YP medium (1% yeast extract and 2% peptone) supplemented with xylose as the sole carbon source. The recombinant strain was aerobically cultivated for 24 h at an agitation speed of 500 rpm and aeration rate of 1 vvm and then shifted to oxygen-limited conditions for ethanol production by adjusting the agitation speed to 300 rpm and the aeration rate to 0.2 vvm [6, 10].

Cell mass was estimated by measuring the absorbance at 600 nm using a spectrophotometer (Hitachi, Japan). The concentrations of glucose, xylose, xylitol, and xylulose were determined by HPLC (Knauer, Germany) with an HPX 87C column (Bio-Rad, U.S.A.). The ethanol concentration was measured by a gas chromatograph (Younglin, Korea) with the Carbowax 20 M column (Supelco, U.S.A.).

The cells were harvested, washed twice in a 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 5 mM β-mercaptoethanol, and disrupted by a Beadbeater (Biospec product, U.S.A.) with glass beads (0.45–0.52 mm) while alternately cooling on ice. The disrupted cell mixture was centrifuged at 10,800 ×g for 10 min to remove the cell debris and glass beads. The supernatant was then used for an analysis of the enzyme activities The xylose reductase activity was measured by monitoring the oxidation of NADPH at 340 nm with a microplate reader (Biotek Instruments, U.S.A.) in a reaction mixture with the following composition: 50 mM potassium phosphate buffer (pH 6.0), 0.1 M xylose, and 0.4 mM NADPH. One unit of xylose reductase activity was defined as the amount of enzyme that oxidized 1 μmol NADPH per minute. The xylitol dehydrogenase activity was measured by monitoring the reduction of NAD^+ at 340 nm with a microplate reader in a reaction mixture with the following composition: 50 mM Tris-HCl buffer (pH 8.5), 8 mM NAD^+, 5 mM $MgCl_2$, and 50 mM xylitol. One unit of xylitol dehydrogenase activity was defined as the amount of enzyme that reduced 1 μmol NAD^+ per minute.

Recombinant *S. cerevisiae* 2805 YIpXR, including the *XYL1* gene in its chromosomal DNA, was grown in YNB medium (0.67% yeast nitrogen base w/o amino acid, 2% glucose) under uracil selection pressure. The specific activity of the XR expressed in the recombinant *S. cerevisiae* 2805 YIpXR was 84 mU/mg protein. The transformant exhibited a 1.2-fold higher xylose reductase (XR) activity than the parent strain, *P. stipitis* CBS 5776, suggesting that the *XYL1* gene was successfully expressed under the control of the glyceraldehyde-3-phosphate promoter in the recombinant *S. cerevisiae*. As reported by Amore *et al.* [1991], xylose fermentations with the *XYL1*-integrated *S. cerevisiae* strain in a xylose-containing medium resulted in no assimilation of xylose due to the lack of xylitol dehydrogenase (XDH) activity. However, this recombinant strain did produce xylitol from xylose when a fermentable carbon source such as glucose was present.

The gene encoding xylitol dehydrogenase (*XYL2*) was introduced into the *XYL1*-integrated *S. cerevisiae* using the episomal plasmid, pY2XDH [9]. The transformed cells were selected on a plate with xylose as the sole carbon source since the untransformed cells, which did not include the *XYL2* gene, could not grow on the plate. The positive clones were identified by Southern blot analysis with a *XYL2* probe using a random primer extension. The transformant containing both *XYL1* and *XYL2* was named *S. cerevisae*

Table 1. Specific activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) in *P. stipitis* and recombinant *S. cerevisiae* strains.

Strains	Specific XR activity (mU/mg protein)	Specific XDH activity (mU/mg protein)
P. stipitis CBS 5776	68	297
S. cerevisiae 2805 YIpXR	84	-
S. cerevisiae 2805 YIpXR:pY2XDH	104	950

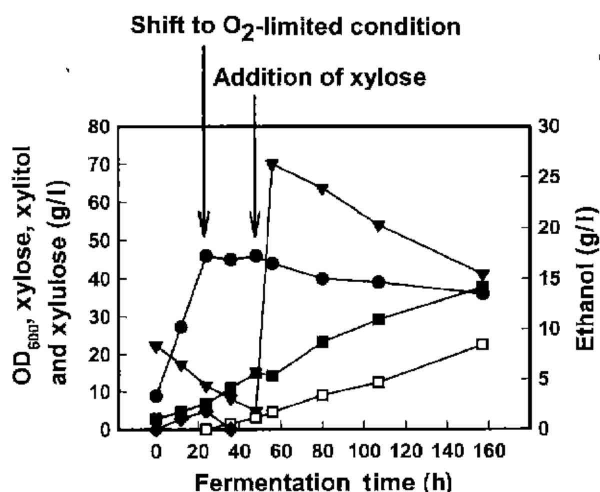

Fig. 3. Fed-batch fermentation of xylose by recombinant *S. cerevisiae* 2805.

YIpXR pY2XDH in YP medium containing 20 g xylose/l at 30°C and pH 5.0. Symbols. ●, optical density at 600 nm; □, ethanol, ▼, xylose, ■ xylitol; ◆, xylulose.

2805 YIpXR:pY2XDH, and was able to express xylitol dehydrogenase as well as xylose reductase (Table 1). The specific activity of xylitol dehydrogenase in the recombinant *S. cerevisiae* 2805 YIpXR:pY2XDH was 3.2-fold higher than that of the parent. Therefore, it would appear that xylose reductase and xylitol dehydrogenase were successfully expressed in the recombinant *S. cerevisiae* strains and that xylose was metabolized to xylulose. It is interesting to note that *S. cerevisiae* YIpXR:pY2XDH expressed slightly more xylose reductase than *S. cerevisiae* YIpXR, even though both strains included the *XYL1* gene in the chromosome.

Xylose fermentation using recombinant *S. cerevisiae* 2805 YIpXR:pY2XDH was performed in a jar fermentor (Fig. 3). *S. cerevisiae* 2805 YIpXR:pY2XDH was able to grow at a specific growth rate of 0.08/h in a growth medium containing xylose as the sole carbon source. The recombinant strain produced 39 g xylitol/l and 4.8 g xylulose/l in aerobic conditions, however, xylulose was consumed later. The production of xylitol increased still further in oxygen-limited conditions. This may be due to the NAD^+ requirement of xylitol dehydrogenase. In aerobic conditions, NADH could be oxidized to NAD^+ in the respiratory chain, however, a low redox balance of NAD^+ to NADH will favor the xylitol dehydrogenase-catalyzed production of xylulose. In oxygen-limited conditions, however, the oxygen supply is insufficient for the regeneration of NAD^+ from glycolysis and the TCA cycle, hence the dehydrogenation of xylitol by xylitol dehydrogenase is limited by the imbalanced redox state, thereby leading to the reduction of xylose to xylitol. Ethanol was only produced under oxygen-limited conditions, indicating that the redox balance regulated by the controlled supply of oxygen facilitated the xylitol dehydrogenase-catalyzed reaction in favor of ethanol production from xylose. Xylose (20 g/l) was consumed within 48 h, therefore, more xylose was supplied. The recombinant yeast was able to produce 8.7 g ethanol/l from xylose with an ethanol yield of 0.13 g ethanol/g xylose and a productivity of 0.054 g ethanol/ $l \cdot h^{-1}$. An analysis of the xylose consumption in the fed-batch fermentation showed that 54% of the xylose consumed were converted to xylitol, 33% to cell mass, and 13% to ethanol. This means that only 46% of the consumed xylose were used for the cell mass and endogenous metabolism. Accordingly, the real ethanol yield was 0.28 g ethanol/g xylose, corresponding to 54% of the theoretical yield estimated from a complete xylose conversion to ethanol. These values are comparable with those reported by other investigations [2, 7, 12, 13]. In this work, a relatively high ethanol yield and productivity were obtained by manipulating the growth conditions favorable for ethanol production to control oxygen supply and intermittent addition of xylose during fermentation.

Acknowledgments

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