



NOTE

Improving xylitol production through recombinant expression of xylose reductase in the white-rot fungus *Phanerochaete sordida* YK-624

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We generated an expression construct consisting of the xylose reductase (XR) gene (*xr*) from *Phanerochaete chrysosporium*. Transformant X7 exhibited increased xylitol production and markedly higher XR activities than the wild-type strain. RT-PCR analysis demonstrated that the increased XR activity was associated with constant expression of the recombinant *xr* gene.

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Lignocellulosic biomass is the most abundant organic compound and has therefore attracted worldwide interest as a feedstock for the production of bioethanol (1,2). For the cost-effective production of value-added products from renewable lignocellulosic resources, microbial bioconversion processes must effectively utilize the pentose sugar xylose, as it is a major component of lignocellulose hydrolysates (3–5). However, the yeast *Saccharomyces cerevisiae*, which is typically used in ethanol production from hexoses, is unable to use pentose sugars such as a xylose (6,7). Therefore, the development of a microbial-based system that can directly utilize pentose is necessary for the economic conversion of lignocellulose in biorefinery processes.

In yeasts and fungi, typically convert xylose to xylulose in a two-step reaction mediated by two kinds of oxidoreductases. An NADPH xylose reductase (XR) first reduces xylose to xylitol, which is then oxidized to xylulose by an NAD-linked xylitol dehydrogenase. And, improvement of xylitol production by recombinant microorganisms was received much attention (4,8). Thus, the simultaneous production of ethanol and xylitol would significantly increase the efficiency of bioethanol production from lignocellulosic biomass.

The white-rot fungus *Phanerochaete sordida* YK-624, which was originally isolated from rotten wood, exhibits greater ligninolytic activity and selectivity than either *Phanerochaete chrysosporium* or *Trametes versicolor* (9). In our previous studies, we successfully developed a superior lignin-degrading strain of *P. sordida* YK-624 using a molecular breeding approach with a homologous expression system (10,11). Here, we used a similar approach to generate high XR gene (*xr*)-expressing transformants of *P. sordida* YK-624

and investigated whether xylitol production was improved in these transformants.

Genomic DNA of *P. chrysosporium* ME-446 was extracted from mycelia using Isopant II (Nippon Gene, Tokyo) and was then used as template for the PCR amplification of the full-length genomic *xr* gene (1144 bp) using the specific primers PcXRF1 and PcXRR1 (Table 1). The obtained PCR product was ligated into the cloning vector pMD20-T (Takara Bio, Shiga, Japan) and introduced into *Escherichia coli* DH5 α for sequencing. Primers XRF1 and XRR1 (Table 1) were designed to amplify the *xr* gene and introduce an *Xba* I site for cloning into the expression plasmid pPsGPD-*pro*, which was generated in our previous study (12). The amplified DNA fragment was digested with *Xba* I and cloned into *Xba* I-digested pPsGPD-*pro*, yielding plasmid pPsGPD-XR.

After pPsGPD-XR was sequenced to verify the absence of PCR errors, it was co-transformed with pPsURA5 using the PEG method into UV-64 protoplasts that were prepared by a standard technique using cellulases (13). Co-transformed clones (X strains) were identified by PCR with the primers gpdF1 and XRR2 (Table 1), which were designed to amplify the recombinant *xr* gene. A total of 13 strains (X1–13) that were co-transformed with pPsGPD-XR and pPsURA5 were obtained.

The effect of recombinant *xr* expression on xylitol production by the X strains was next investigated. *P. sordida* YK-624 (WT) and the X strains were incubated on PDA plates at 30°C for 3 days, and 10-mm diameter disks were then punched out from the growing edge of the mycelia using a sterile cork borer. Two mycelial disks for each strain were placed into a 100-mL Erlenmeyer flask containing 10 mL nitrogen-limited Kirk medium (14) supplemented with 1.5% xylose as a carbon source, and the flask was then statically incubated at 30°C for 7 days. Xylitol and xylose concentrations were determined using a high-performance liquid chromatograph equipped with a Shodex SH1821 column (8.0 mm $\times$ 300 mm, Showa Denko K.K., Tokyo, Japan) at 75°C with 0.5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL min^{−1}. Xylitol and xylose were

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TABLE 1. Oligonucleotides used as primers in this study.

Primer name	Nucleotide sequence (5'-sequence-3')
PcXRF1	ATGCTTTCTTCGCCAACCTC
PcXRR1	TCAGCAITTCACGGAGAAAGTACG
XRF1	AATCTAGAATGCTTTCTTCGCCAACCTC
XRR1	AATCTAGATCAGCAITTCACGGAGAAAGTACG
gpdF1	AAGCAGCGAGGATTGTACC
XRR2	GATGGAAGGTGTTCCACAG
ActinF	AGCACGGTATCGTCACCAAC
ActinR	AGCGAAACCCTCGTAGATGG

detected using an online refractive index detector. Transformant strain X7 exhibited the highest xylitol production among the 13 transformant strains (data not shown) and was therefore selected for subsequent analyses.

We next analyzed the transcription levels of *xr* by RT-PCR and measured the XR activity of strain X7 during 8 days of culture in nitrogen-limited Kirk medium. Mycelial samples were collected and stored at -80°C . Total RNA was isolated from the thawed samples using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit (Takara Bio) and the gene-specific primer sets gpdF1-XRR2 and ActinF-ActinR (Table 1).

For the measurement of XR activity, each mycelial sample was added to 2 ml of 250 mM phosphate buffer (pH 7.0) and was then homogenized using a Polytron PT1200E (Kinematica, Canada) at 4°C . The homogenate was centrifuged (4°C , $10,000\times g$, 10 min) and the obtained supernatant was used as a cell-free extract for the measurement of XR activity, which was determined by monitoring the oxidation of NADPH to NADP^{+} ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) (15). The reaction mixture (1 mL) consisted of 10 mM 2-mercaptoethanol, 50 mM xylose, and 0.17 mM NADPH in 250 mM phosphate buffer (pH 7.0).

The time course of xylitol production by strains X7 and WT over 2, 4, 6, and 8 days of incubation in nitrogen-limited Kirk medium was monitored. After 4 days of incubation, xylitol production by strain X7 reached 1.39 g L^{-1} , whereas the WT strain had only produced 0.61 g L^{-1} (Fig. 1). In contrast to the WT strain, the production of xylitol by strain X7 continued to increase, reaching 3.61 g L^{-1} of xylitol after 8 days of incubation (Fig. 1). We next evaluated the consumption rates by the xylose consumption, and the xylose consumption rate of the strain X7 (31.8%) was higher than that of WT strain (11.2%).

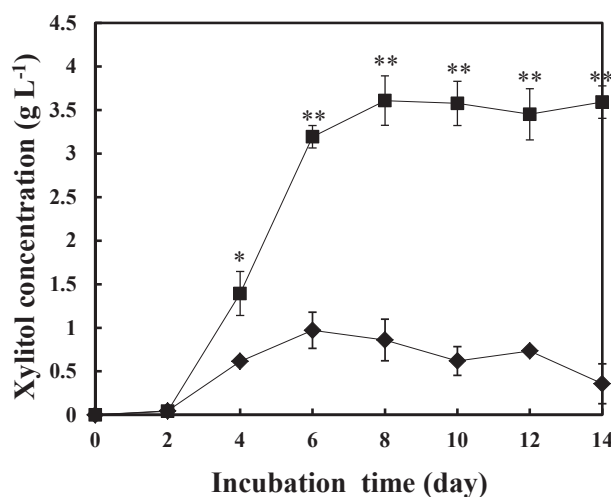


FIG. 1. Time course of xylitol production by the wild-type (WT) strain (diamonds) and strain X7 (squares). Values are the means $\pm$ SD of triplicate samples. Asterisks indicate values that were determined by the Student's *t*-test to be significantly different from WT (* $P < 0.05$, ** $P < 0.01$).

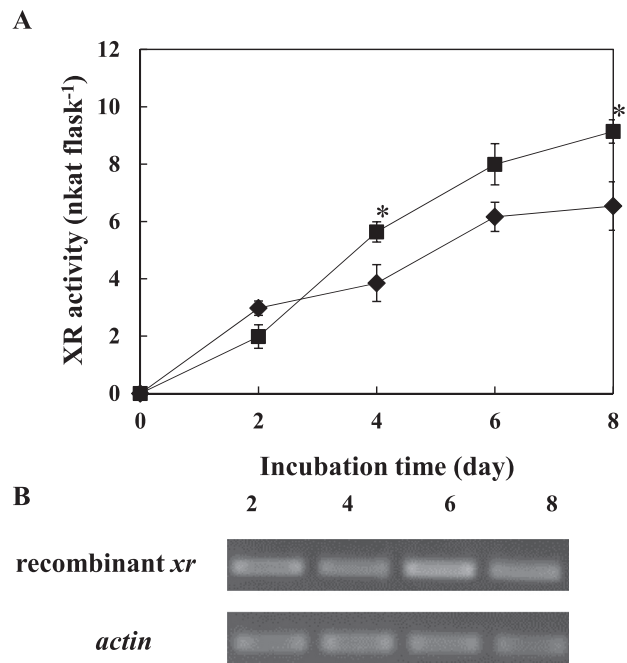


FIG. 2. XR activity and expression of the *xr* gene by strain X7. (A) Time course of XR activities in the wild-type strain (diamonds) and strain X7 (squares). Values are the means $\pm$ SD of triplicate samples. Asterisks indicate values that were determined by the Student's *t*-test to be significantly different from WT (* $P < 0.05$). (B) RT-PCR analysis of *xr* gene transcription in strain X7. The expression of actin was used as an internal control.

XR activity of strain X7 increased gradually for 8 days and was higher than that of WT throughout the incubation period (Fig. 2A). The highest XR activity for strain X7 was $9.4 \text{ nkat flask}^{-1}$ after 8 days of incubation, whereas that for WT was $6.5 \text{ nkat flask}^{-1}$ (Fig. 2A). The difference of fungal grown in WT strain and strain X7 was not observed under glucose- or xylose-containing medium. Consistent with these findings, RT-PCR indicated that strain X7 had constant *xr* expression between days 2–8 (Fig. 2B). Taken together, these results suggest that the increase of XR activity in strain X7 was due to constant expression of the recombinant *xr* gene, and that the recombinant expression of *xr* in the white-rot fungus *P. sordida* YK-624 improves xylitol production.

To conclude, the recombinant expression of *xr* is effective for the improvement of the xylitol production in white-rot fungi. Recently, direct ethanol production by the white-rot fungus *Phlebia* sp. MG-60 in pure culture without the addition of exogenous chemicals or enzymes was reported (16). In future studies, we intend to develop a molecularly bred strain of white-rot fungus that can simultaneously produce ethanol and xylitol from woody biomass with high yields.

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