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Note

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

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Running title: Improving xylitol production by XR gene expression

Abstract

We generated an expression construct consisting of the xylose reductase (XR) gene (*xr*) from *P. 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.

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 *P. 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 ISOPLANT 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. The obtained PCR product was ligated into the cloning vector pMD20-T (Takara Bio, Shiga, Japan) and introduced into *Escherichia coli* DH5α for sequencing. Primers XRF1 and XRR1 were designed to amplify the *xr* gene and introduce an *Xba* I site for cloning into the expression plasmid p*PsGPD-pro*, which was generated in our previous study (12). The amplified DNA fragment was digested with *Xba* I and cloned into *Xba* I-digested p*PsGPD-pro*, yielding plasmid p*PsGPD*-XR.

After p*PsGPD*-XR was sequenced to verify the absence of PCR errors, it was co-transformed with p*PsURA5* using the PEG method into UV-64 protoplasts that were prepared by a standard technique using cellulases (13)**.** Co-transformed clones (X

61 strains) were identified by PCR with the primers gpdF1 and XRR2, which were
62 designed to amplify the recombinant *xr* gene. A total of 13 strains (X1-13) that were
63 co-transformed with p*PsGPD*-XR and p*PsURA5* were obtained.
64 The effect of recombinant *xr* expression on xylitol production by the X strains was
65 next investigated. *P. sordida* YK-624 (WT) and the X strains were incubated on PDA
66 plates at 30 °C for 3 days, and 10-mm diameter disks were then punched out from the
67 growing edge of the mycelia using a sterile cork borer. Two mycelial disks for each
68 strain were placed into a 100-mL Erlenmeyer flask containing 10 mL nitrogen-limited
69 Kirk medium (14) supplemented with 1.5% xylose as a carbon source, and the flask was
70 then statically incubated at 30 °C for 7 days. Xylitol and xylose concentrations were
71 determined using a high-performance liquid chromatograph equipped with a Shodex
72 SH1821 column (8.0 mm × 300 mm, Showa Denko K.K., Tokyo, Japan) at 75 °C with
73 0.5 mM $H_2SO_4$ as the mobile phase at a flow rate of 0.6 mL $min^{-1}$. Xylitol and xylose
74 were detected using an online refractive index detector. Transformant strain X7
75 exhibited the highest xylitol production among the 13 transformant strains (data not
76 shown) and was therefore selected for subsequent analyses.
77 We next analyzed the transcription levels of *xr* by RT-PCR and measured the XR
78 activity of strain X7 during 8 days of culture in nitrogen-limited Kirk medium. Mycelial

79 samples were collected and stored at -80 °C. Total RNA was isolated from the thawed
80 samples using an RNeasy Plant Mini kit (Qiagen, Valencia, CA). RT-PCR was
81 performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit (Takara Bio) and
82 the gene-specific primer sets gpdF1-XRR2 and ActinF-ActinR.
83 For the measurement of XR activity, each mycelial sample was added to 2 ml of
84 250 mM phosphate buffer (pH 7.0) and was then homogenized using a Polytron
85 PT1200E (Kinematica, Canada) at 4 °C. The homogenate was centrifuged (4 °C, 10,000
86 x *g*, 10 min) and the obtained supernatant was used as a cell-free extract for the
87 measurement of XR activity, which was determined by monitoring the oxidation of
88 NADPH to $NADP^+$ ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$) (15). The reaction mixture (1 ml) consisted
89 of 10 mM 2-mercaptoethanol, 50 mM xylose, and 0.17 mM NADPH in 250 mM
90 phosphate buffer (pH 7.0).
91 The time course of xylitol production by strains X7 and WT over 2, 4, 6, and 8
92 days of incubation in nitrogen-limited Kirk medium was monitored. After 4 days of
93 incubation, xylitol production by strain X7 reached 1.39 g $L^{-1}$, whereas the WT strain
94 had only produced 0.61 g $L^{-1}$ (Fig. 1). In contrast to the WT strain, the production of
95 xylitol by strain X7 continued to increase, reaching 3.61 g $L^{-1}$ of xylitol after 8 days of
96 incubation (Fig. 1). We next evaluated the consumption rates by the xylose

97 consumption, and the xylose consumption rate of the strain X7 (31.8%) was higher than
98 that of WT strain (11.2%).
99 XR activity of strain X7 increased gradually for 8 days and was higher than that of
100 WT throughout the incubation period (Fig. 2a). The highest XR activity for strain X7
101 was 9.14 nkat flask$^{-1}$ after 8 days of incubation, whereas that for WT was 6.54 nkat
102 flask$^{-1}$ (Fig. 2a). The difference of fungal grown in WT strain and strain X7 was not
103 observed under glucose- or xylose-containing medium. Consistent with these findings,
104 RT-PCR indicated that strain X7 had constanrt *xr* expression between days 2 to 8 (Fig.
105 2b). Taken together, these results suggest that the increase of XR activity in strain X7
106 was due to constant expression of the recombinant *xr* gene, and that the recombinant
107 expression of *xr* in the white-rot fungus *P. sordida* YK-624 improves xylitol production.
108 To conclude, the recombinant expression of *xr* is effective for the improvement of
109 the xylitol production in white-rot fungi. Recently, direct ethanol production by the
110 white-rot fungus *Phlebia* sp. MG-60 in pure culture without the addition of exogenous
111 chemicals or enzymes was reported (16). In future studies, we intend to develop a
112 molecularly bred strain of white-rot fungus that can simultaneously produce ethanol and
113 xylitol from woody biomass with high yields.
114

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Table 1 Oligonucleotides used as primers in this study.

| Primer name | Nucleotide sequence (5'-sequence-3') |
|---|---|
| PcXRF1 | ATGCTTTCTTCGCCAACCCTC |
| PcXRR1 | TCAGCATTCACGGAGAAAGTACG |
| XRF1 | AATCTAGAATGCTTTCTTCGCCAACCCTC |
| XRR1 | AATCTAGATCAGCATTCACGGAGAAAGTACG |
| gpdF1 | AAGCAGCGAGGATTGTACC |
| XRR2 | GATGGAAGGTGTTCCACAG |
| ActinF | AGCACGGTATCGTCACCAAC |
| ActinR | AGCGAAACCCTCGTAGATGG |

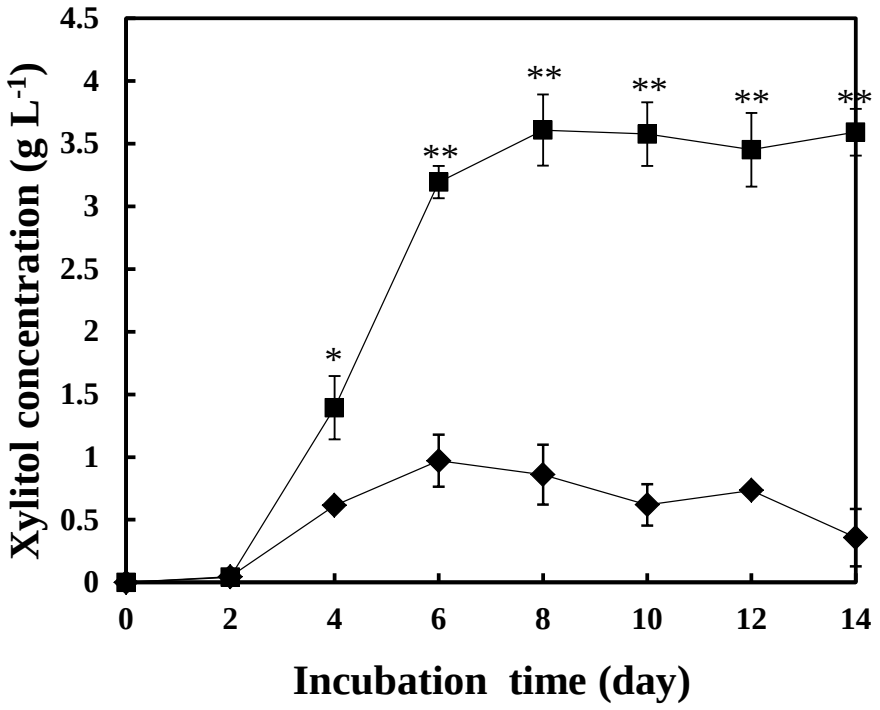


Fig. 1 Time course of xylitol production by the wild-type (WT) strain (closed diamonds) and strain X7 (closed squares). Values are the means ± SD of triplicate samples. Asterisks indicate values that were determined by the Student's t-test to be significantly different from WT (*$P$<0.05, **$P$<0.01).

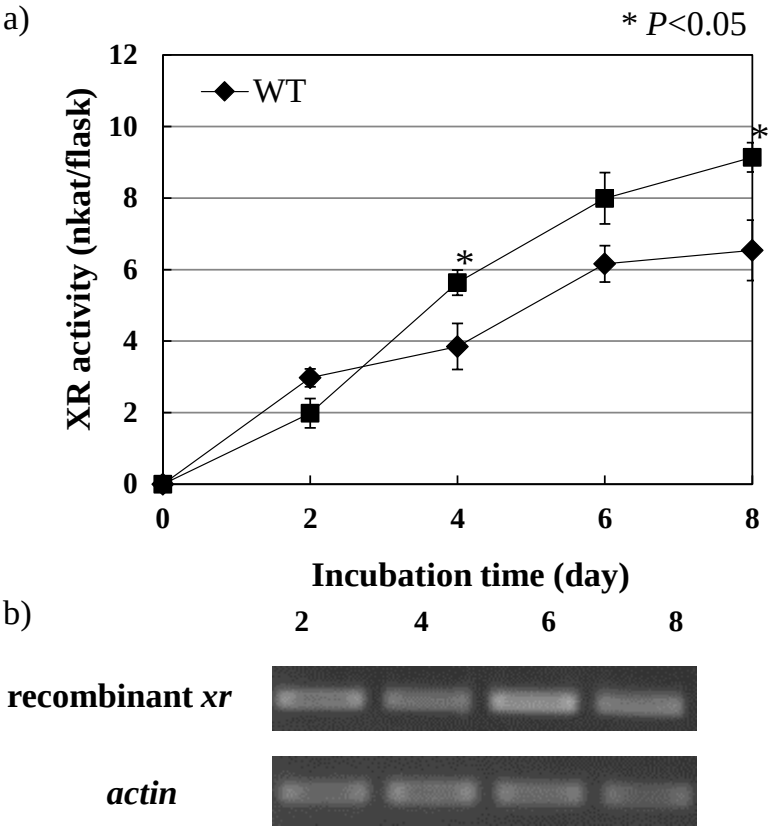


Fig. 2 XR activity and expression of the *xr* gene by strain X7. a) Time course of XR activities in the wild-type strain (closed diamonds) and strain X7 (closed squares). Values are the means ± SD of triplicate samples. Asterisks indicate values that were determined by the Student's t-test to be significantly different from WT (**P*<0.05, ***P*<0.01). b) RT-PCR analysis of *xr* gene transcription in strain X7. The expression of *actin* was used as an internal control.