Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2017

www.elsevier.com/locate/jbiosc

Metabolic pathway analysis of the xylose-metabolizing yeast protoplast fusant ZLYRHZ7

Jingping Ge,[1,‡] Renpeng Du,[1,2,‡] Gang Song,[1] Yuhuan Zhang,[1] and Wenxiang Ping[1,*]

Key Laboratory of Microbiology, College of Life Science, Heilongjiang University, Harbin 150080, PR China[1] *and School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, PR China*[2]

Received 8 September 2016; accepted 20 April 2017
Available online xxx

Xylose is the second major fermentable sugar present in hard woods and herbs (after D-glucose). Therefore, efficient conversion of xylose to ethanol is essential for the commercialization of lignocellulosic ethanol, which may provide an ideal alternative to fossil fuels in the future. ZLYRHZ7 is a fusant produced by protoplast fusion between two different yeast species, *Saccharomyces cerevisiae* W5 and *Candida shehatae* 20335, which is able to utilize xylose to produce ethanol. To improve ethanol production and to quantitatively analyze metabolic pathway in ZLYRHZ7, we used high performance liquid chromatography (HPLC) to assess the utilization rates of xylose, xylitol, and xylulose, and to measure ethanol yields using xylose, xylitol, and xylulose as sole carbon sources. The ethanol yields reached 0.549 ± 0.003, 0.567 ± 0.003 and 0.544 ± 0.005 g/g in 72 h, which indicated that the metabolic pathways from xylose to xylitol, xylitol to xylulose, and xylulose to ethanol, respectively, were functional. In addition, enzyme activity and qRT-PCR analyses showed that the xylose metabolism-related enzymes xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulose kinase (XK) and their respective genes were expressed at significantly higher levels in ZLYRHZ7 than in both *S. cerevisiae* W5 and *C. shehatae* 20335 at 24, 48, and 72 h of fermentation. These results clearly show that the fusant ZLYRHZ7, obtained by protoplast fusion of two different yeast species, has the ability to ferment xylose to produce ethanol.

[**Key words:** Yeast fusant; ZLYRHZ7; Xylose fermentation; Bioethanol production; Gene expression]

With the continuing and unsustainable consumption of fossil energy, the development of sources of clean and renewable energy has become a priority in many parts of the world (1). Because it has the advantages of being clean, renewable, and easy to transport, ethanol has been identified as the most promising form of renewable energy (2). Ethanol is a primary alcohol derived from fermentation of the carbohydrate or sugar fractions of biomass materials. In the last few years, considerable effort has been focused on ethanol production from lignocelluloses (3). Using lignocellulose containing sources of biomass to produce ethanol will not only broaden the raw material sources for producing fuel ethanol, but can also help alleviate environmental pollution caused by agricultural and forestry wastes (4). Hydrolysis of lignocellulosic feedstock can produce a large amount of monosaccharide sugars, with glucose and xylose being the two major monosaccharides present in lignocellulose hydrolysates (5,6). Therefore, to realize the most efficient conversion of lignocellulose to ethanol, xylose utilization is particularly important. In nature, approximately 100 kinds of microorganisms can utilize xylose, and of these, *Saccharomyces cerevisiae* has been widely used in industrial production (7,8) mainly because of its predominant status in ethanol fermentation and its high tolerance to ethanol and inhibitors. However, *S. cerevisiae* cannot effectively utilize xylose, and the fermentation properties of yeast strains can thus not meet industrial requirements (9,10).

Utilization of xylose in other yeasts occurs through a three-step oxidoreductive pathway consisting of xylose reductase (XR, EC 1.1.1.21), xylitol dehydrogenase (XDH, EC 1.1.1.9), and xylulokinase (XK, EC 2.7.1.17), that achieves net isomerization of xylose into ethanol via xylitol as an intermediate (11,12). Xylose can be converted into xylulose by introducing *XYL1*, which encodes NADPH-linked XR, and *XYL2*, which encodes NAD-linked XDH, from the xylose fermenting yeasts *Pichia stipitis* or *Candida shehatae* (13,14). Thus, the frequently used recipient yeast *S. cerevisiae* can express other xylose metabolism genes so as to adapt to the requirements of industrial ethanol production (15).

Using protoplast fusion between *S. cerevisiae* W5 and *C. shehatae* 20335, our team has successfully constructed a yeast fusant strain known as ZLYRHZ7 (16). However, whether the fusant ZLYRHZ7 can be used for industrial production, and whether the xylose metabolism pathway is intact, remain to be verified. In this study, we initially sought to verify that the xylose metabolism pathway in ZLYRHZ7 remained functional using different intermediate metabolites as sole carbon source for fermentation (17,18). Furthermore, the gene expression levels and enzyme activities for XR, XDH, and XKS were quantified using polymerase chain reaction (PCR), quantitative real-time PCR (qRT-PCR) and enzyme assays. This research provides a foundation for constructing recombinant strains that are highly efficient at fermenting xylose, and also

* Corresponding author. Tel.: +86 0451 86609106; fax: +86 0451 86608046.
E-mail address: wenxiangp@aliyun.com (W. Ping).
‡ The first two authors contributed equally to this work and share the first authorship.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.04.016

provides a reference for utilizing lignocellulosic biomass as the raw material to produce bio-ethanol (19).

MATERIALS AND METHODS

Yeast strains ZLYRHZ7 was derived from the protoplast fusion of *S. cerevisiae* W5 and *C. shehatae* 20335. *S. cerevisiae* W5, a diploid wild-type strain, was isolated from soil collected in Heilongjiang Province, China. *C. shehatae* ACCC 20335 was purchased from the Agricultural Culture Collection of China (ACCC), and all strains are maintained in our laboratory (16). The two yeast strains were grown in yeast extract-peptone-dextrose (YEPD) liquid medium (2% peptone, 1% yeast extract, and 2% dextrose; w/v) or in yeast extract-peptone-xylose (YEPX) liquid medium (2% peptone, 1% yeast extract, and 2% xylose; w/v).

Growth conditions and fermentation methods For the seed culture, a single colony was inoculated into 20 mL/50 mL YEPD liquid medium and incubated at 30°C, overnight. Yeasts are facultative anaerobic organisms, which require oxygen during the fermentation process. A pinhole stopper was chosen to control fermentation conditions in the flasks based on the level of dissolved oxygen, ethanol yield, and biomass production (data not shown) (19). For the determination of metabolic pathways, the seed cultures were harvested and resuspended in distilled water for 2 h at 30°C and shaken at 140 rpm to obtain starvation cells. The starvation cells were then inoculated into xylose, xylitol, and xylulose medium at a concentration of 5% (*v*/*v*), using 125 mL working volumes in 250 mL flasks with a pinhole stopper. Cultures were shaken at 200 rpm for 72 h at 30°C prior to measuring the ethanol yield and the use of different metabolic intermediate carbon sources. All of the products were detected by high performance liquid chromatography (HPLC) (20).

Xylose fermentation medium consisted of 1% D-(+)-xylose, 0.25% $(NH_4)_2SO_4$, 0.25% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$ and 0.025% $CaCl_2$ (*w*/*v*; pH 5.0); xylitol and D-xylulose fermentation media contained 1% xylitol or D-xylulose instead of D-(+)-xylose (*w*/*v*; pH 5.0). All reagents were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd., China.

HPLC analysis Batch fermentation with xylose, xylitol, and xylulose showed that ethanol was produced from xylose under microaerobic conditions. To investigate the conversion of xylose to ethanol, microaerobic batch fermentations of recombinant ZLYRHZ7 and the parent strains *S. cerevisiae* W5 and *C. shehatae* 20335 (cell dry weights were 0.256 ± 0.006, 0.239 ± 0.011, and 0.205 ± 0.008 g/L, respectively) were carried out using defined media containing xylose, xylitol, or xylulose as the sole carbon source. Untransformed yeast strains W5 and 20335 were used as controls. The supernatants were sampled every 24 h during 72 h of fermentation for analysis of the ethanol production by HPLC (Shimadzu LC-20A) using an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm; Bio-Rad Laboratories, Inc.) at 65°C with a refractive index detector (RID-10A). The eluent used was 5 mM H_2SO_4 at a low flow rate of 0.8 mL/min. The analysis time was 18 min. Sample injection volume was 20 μL. Commercially available ethanol (Tianjin Guangfu Technology Development Co., China), xylose (Shanghai Boao Biotechnology Co., China), xylitol (Tianjin Kemiou Chemical Reagents Co.), and xylulose (Institute of Guangfu Fine Chemical Industry of Tianjin) were used as standards.

For batch fermentation with xylose as a sole carbon source, recombinant cells that had been pre-cultured in YEPX were harvested by centrifugation at 13,000 ×*g* for 10 min and inoculated into a 500 mL baffled flask containing 300 mL medium. Culture temperature and agitation speed were maintained at 30°C and 200 rpm, respectively, in a shaking incubator. For batch fermentation with xylulose and xylitol as sole carbon source, recombinant cells were pre-cultured in YEPX medium, and grows as described above.

PCR analysis A single colony growing on the medium was inoculated into 20 mL/50 mL YEPX liquid medium and incubated overnight at 30°C with shaking at 140 rpm to assay for the presence of the xylose metabolic genes, *XYL1*, *XYL2*, and *XKS* every 24 h. Using ZLYRHZ7 genomic DNA extracted with the TIANamp Yeast DNA Kit (DP307-02, Tiangen Biotech Co., Beijing, China) as template, amplification reactions for XR, XDH, and XK, were performed with a Mastercycler gradient cycler (Eppendorf GA, Germany) under the following conditions: reactions contained 0.2 μL *Taq* DNA polymerase (5 U/mL), 0.8 μL dNTP mixture (2.5 mmol/L), 0.8 μL $MgCl_2$ (25 mmol/L), 1 μL PCR buffer (10×, Mg^{2+} free), 1 μL each primer (1 pmol/mL) and 2 μL DNA template in a total volume of 10 μL. The sequences of the primers were as follows. YX-*XYL1*-1 and YX-*XYL1*-2: 5′-ACTTCTAGATACATCCACAATGAGCCC-3′ and 5′-TTCGGATCTCTACGCAAAGAAAGCAG-3′, respectively, for *XYL1* (primers designed from the *XYL1* sequence of *C. shehatae*); ZMY-*XYL2*-1 and ZMY-*XYL2*-2: 5′-CCTACTAGTA-TGACTGCTAACCCTTCGCTC-3′ and 5′-CCGACTAGTCTATTCAGGGCCATCAATGAAAC-3′, for *XYL2* (primers designed from the *XYL2* sequence of *C. shehatae*); and CXS-*XKS1*-1 and CXS-*XKS1*-2: 5′-CGGACTAGTAGTACTTTAATGTTGTGTT-CAGTAA-3′ and 5′-CGCGTCGACTTTAGATGAGAGTCTTTTCCAG-3′, for *XKS* (primers designed from the *XKS* sequence of *S. cerevisiae*). The PCR conditions were as follows: a preliminary step of 7 min at 94°C; followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, with a final extension step of 10 min at 72°C. Amplified DNA fragments were examined by electrophoresis of 5 μL samples of each PCR on 1% agarose gels (21).

RNA isolation and qRT-PCR Total RNA was extracted from yeast strains ZLYRHZ7, W5, and 20335 using the RNAprep Pure Bacteria Kit (Tiangen Biotech Co. Ltd.) following the manufacturer's instructions. The concentration and quality of the RNA samples were determined using spectrophotometry (Shanghai Spectrum Instruments Co. Ltd., China) by measuring the absorbance ratio at 260 nm/280 nm ($A_{260/280}$) and by gel electrophoresis. After 42°C treatment for 2 min to remove genomic DNA contamination, first-strand cDNA was synthesized from 1 μg samples of RNA by reverse transcription using the BioRT cDNA First Strand Synthesis Kit (Bioer Technology, China) as directed by the manufacturer. A 10-fold dilution series of cDNA was prepared in triplicate for qRT-PCR analysis. The relative expression of genes in different strains were determined with at least 3 biological replicates using SYBR Green-based detection in triplicate on a 7500 Real-Time PCR System (Applied Biosystems Inc., USA). The florescence signals were measured during the annealing period of each cycle and the signal intensity was used to calculate amplification curves. The 18S rRNA gene was chosen to normalize RNA amounts as an internal control. The suitability of this 18S rRNA gene was verified by isolation of both genomic DNA and RNA. The expression ratio of isolated RNA and DNA was shown to be constant throughout the experiment.

The qRT-PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and then 95°C for 1 min. All reactions were performed in 96-well reaction plates. Fluorescence data collected during the final 95°C step were analyzed with the ABI 7500 Analysis Software. The sequences of the primers were as follows: 18S (F) 5′-TTGGAGGGCAAGTCTGGT-3′ and 18S (R) 5′-GGAAAGGCTCGGT-TAGGT-3′ for the 18S rRNA gene; YX-*XYL1*-1 5′-ACCAGGTCTACAACGCCATC-3′ and YX-*XYL1*-2 5′-TCTCCTCGATTGGGACAAAC-3′ for *XYL1*; ZMY-*XYL2*-1 5′-CGCCATT-GAGCCAGGTAT-3′ and ZMY-*XYL2*-2 5′-TCGGTGGAGTTTGGAGTG-3′ for *XYL2*; and CXS-*XKS1*-1 5′-GATGCCTGTGGTATGAAC-3′ and CXS-*XKS1*-2 5′-GTTTGGCA-GAGTTGGATG-3′ for *XKS*. The expression levels of all candidate reference genes were detected by qRT-PCR in each cDNA sample. Diluted cDNA equivalent to 1 ng RNA starting material was used as a template for qRT-PCR amplification. The relative expression of the *XYL1*, *XYL2*, and *XKS* genes were normalized to the transcriptional level of the 18S rRNA. The relative quantification of gene transcription levels in the different samples were calculated based on Ct values as described previously (22).

Enzyme assays Cell-free extracts for the assays of xylose metabolic enzymes were prepared as follows. The fusant strain ZLYRHZ7 was cultivated in YEPX medium overnight at 30°C. For determination of XR, XDH, and XK activities, the cells were collected by centrifugation at 16,000 rpm at room temperature, and the pellets were suspended in 100 mmol/L phosphate buffer (pH 7.0). After adding 0.2 g of glass beads to the cell suspension, the mixture was vortexed vigorously for 1 min and then cooled for 4 min, and this treatment was repeated three times. After centrifugation at 16,000 rpm for 10 min at 4°C, the supernatant containing the protein crude extract was used for the enzyme assays. The reaction mixture for XR contained 100 mmol/L potassium phosphate buffer (pH 7.4), 0.15 mmol/L NADPH or NADH, and 0.2 mmol/L xylose. For XDH, the reactions contained 0.1 mmol/L Tris−HCl buffer (pH 7.0), 1 mmol/L $MgCl_2$, 5 mmol/L NAD^+, and 50 mmol/L xylitol. Based on a previous report, XK was assayed in a reaction mixture consisting of 500 mmol/L Tris−HCl buffer (pH 7.8), 2 mmol/L NADH, 10 mmol/L ATP, 50 mmol/L $MgCl_2$, 10 mmol/L xylulose, 10 mmol/L PEP-K, and PK (40.3 U/L)-LDH (67.5 U/L) (13,22). In all cases, the absorbance at 340 nm was monitored with a UV−vis spectrophotometer after addition of the crude enzyme solution. Reactions were carried out at 30°C in triplicate. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of NADPH or NADH, or reducing 1 μmol of NAD^+ per minute in the reaction conditions described above (13).

Statistical analyses The data are presented as the means ± standard errors of three independent experiments. Average ± standard errors of all obtained data were defined. JMP software (SAS Institute Inc., version 9.0.2) was used for the statistical analysis, and Tukey's test was performed for determining the significant differences at 95% confidence interval ($p < 0.05$).

RESULTS

Ethanol production by ZLYRHZ7 using different carbon sources Xylose could be used as the sole carbon source during 72 h of fermentation, which indicated that ZLYRHZ7 can utilize xylose for the production of ethanol. In *S. cerevisiae* W5, *C. shehatae* 20335, and ZLYRHZ7, xylose utilization, the xylose utilization rate, ethanol reduction, ethanol yield, and xylitol production showed differences ($p < 0.05$). This agrees with the results of a previous study (data not shown) (16). Thus, ZLYRHZ7 can utilize xylose to produce higher amounts of ethanol, and the metabolic pathway from xylose to xylitol is intact in the fusant.

ZLYRHZ7, 20335, and W5 consumed 8.651 ± 0.236, 7.892 ± 0.162, and 2.511 ± 0.117 g/L of xylitol, respectively, when xylitol was used as the sole carbon source after 72 h of

Please cite this article in press as: Ge, J., et al., Metabolic pathway analysis of the xylose-metabolizing yeast protoplast fusant ZLYRHZ7, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.04.016