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BIOENERGY AND BIOFUELS

An efficient xylose-fermenting recombinant *Saccharomyces cerevisiae* strain obtained through adaptive evolution and its global transcription profile

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Abstract Factors related to ethanol production from xylose in engineered *Saccharomyces cerevisiae* that contain an exogenous initial metabolic pathway are still to be elucidated. In the present study, a strain that expresses the xylose isomerase gene of *Piromyces* sp. *Pi-xylA* and overexpresses *XKS1*, *RPE1*, *RKI1*, *TAL1*, and *TKL1*, with deleted *GRE3* and *COX4* genes was constructed. The xylose utilization capacity of the respiratory deficiency strain was poor but improved via adaptive evolution in xylose. The μ_{max} of the evolved strain in 20 gl^{-1} xylose is 0.11±0.00 h^{-1}, and the evolved strain consumed 17.83 gl^{-1} xylose within 72 h, with an ethanol yield of 0.43 gg^{-1} total consumed sugars during glucose–xylose cofermentation. Global transcriptional changes and effect of several specific genes were studied. The result revealed that the increased xylose isomerase acivity, the upregulation of enzymes involved in glycolysis and glutamate synthesis, and the downregulation of trehalose and glycogen synthesis, may have contributed to the improved xylose utilization of the strain. Furthermore, the deletion of *PHO13* decreased the xylose growth in the respiration deficiency strain although deleting *PHO13* can improve the xylose metabolism in other strains.

Keywords Xylose isomerase · Respiratory deficiency · Ethanol · *PDC6* · *PHO13* · *CWP1*

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Introduction

Saccharomyces cerevisiae is a promising organism for lignocellulosic ethanol fermentation. It effectively ferments glucose, fructose, and mannose through glycolysis (van Maris et al. 2006). Xylose is the second predominant sugar in plant biomass after glucose. Converting xylose into ethanol using *S. cerevisiae* presents an economic interest (Hahn-Hägerdal et al. 2007; Liu et al. 2010). However, *S. cerevisiae* cannot naturally utilize xylose, although genes that encode the required metabolic enzymes are included in its genome (Hahn-Hägerdal et al. 2007; Jeffries and Jin 2004; Wang et al. 2004). Metabolic engineering was performed to construct the xylose-utilizing strain by introducing the heterogeneous xylose metabolic pathway, which was xylose reductase-xylitol dehydrogenase (XR-XDH) or xylose isomerase (XI). The xylose-utilizing capacity of recombinant strain then further optimized by enhancing the downstream metabolic pathway such as xylulokinase (XK) and the pentose phosphate pathway (PPP). Moreover, to decrease the by-production of xylitol, the aldose reductase (encoded by the *GRE3* gene) was destroyed and the redox state of XR-XDH-expressing strain was balanced via various cofactor engineering (Eliasson et al. 2000b; Hahn-Hägerdal et al. 2007; Jeffries and Jin 2004; Karhumaa et al. 2007; Kuyper et al. 2005a; Peng et al. 2012). However, the xylose-fermenting efficiency of strain, which was obtained using only these rational manipulations, is less than expected.

Evolutionary engineering can develop strain with expected phenotypes based on the principles of mutation and selection (Sauer 2001). This strategy has been applied successfully by various groups in improving xylose fermentation. Sonderegger and Sauer (2003) developed a *Ps*-XR-XDH-expressing strain using EMS mutation and adaptive cultivation. The mutant, TMB3001C5, achieved a ~50 % higher specific xylose utilization rate and produced 19 % more ethanol. Kuyper et al. (2005b, 2004) and Wisselink et al. (2009) reported an evolved

strain that is capable of cofermenting glucose, xylose, and arabinose faster and more effectively through cycles of adaptive batch and chemostat cultivation.

To understand the molecular mechanism of enhanced xylose metabolism and provide new guidelines for further strain modification, the transcriptional profile of the evolved strain and its response to xylose were investigated in XR-XDH expressing strains (Jin et al. 2004; Salusjärvi et al. 2006; Sonderegger et al. 2004). Meanwhile, the transcriptomic data for the XR-XDH evolved strain exhibited a respiratory response (Jin et al. 2004). The conversion of xylose into ethanol in the XI-expressing strain does not require cofactors for redox metabolic balance. Therefore, it can be a good model for studying the mechanism underlying the metabolic regulation of xylose fermentation in *S. cerevisiae*.

In the present work, an effective xylose-fermenting strain was obtained by adaptive evolution based on the strain using techniques, such as the expression of the xylose isomerase of *Piromyces* sp. (*Pi-xylA*) and enhancement of the downstream metabolic pathway of xylose. Then, the global transcriptional profile of the evolved strain was compared with the reference strain. Several significantly regulated genes were tested for their effect on xylose metabolism.

Materials and methods

The construction of plasmids and strains

The yeast expressional plasmid pJFE3 (Fig. 1) was constructed by introducing the *TEF1* promoter (*TEF1p*) and the *PGK1* terminator (*PGK1t*) in the YEplac195 vector (Gietz and Akio 1988). Then, the xylose isomerase gene from *Piromyces* sp. (*Pi-xylA*, GenBank accession no. CAB76571.1) was artificially synthesized and cloned into pJFE3 under the control of *TEF1p* and *PGK1t* to generate the pJX5 plasmid (Fig. 1). The plasmid pJX5 was transformed into the respiratory-deficient strain BSPC042 (Peng et al. 2012), to produce the strain BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*; Table 1).

Gene deletion was preformed as previously described (Peng et al. 2012) and was confirmed by PCR. The selection marker, but not the deleted gene sequence, was replicated from the genome of strains. The integrating vector pYMIK with the *PGK1* promoter (Wang et al. 2004) was employed for the overexpression procedure, and the transformants were confirmed by reverse transcription polymerase chain reaction (RT-qPCR). The plasmids and the *S. cerevisiae* strains used in this work are listed in Table 1.The primers used for PCR are listed in Table S1 in the Electronic supplementary material (ESM).

Adaptive evolution of strain on xylose

The adaptive evolution process was performed according to the methods of Peng et al. (2012) until the biomass doubling time for xylose did not show significant shortening. Several single clones were isolated and evaluated. One of the fastest growing clones was selected and designated as BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE). The abbreviation AE was used to indicate the strain after adaptive evolution.

Batch fermentation

Minimal medium (6.7 gl^{-1} YNB, pH 6.0) with different glucose and/or xylose concentrations was used for batch fermentation. The cells were grown at 30 °C and 200 rpm. A cotton plug and a rubber stopper were used in the shake flasks for aerobic and air-limited cultivation conditions, respectively.

Measurement of enzymatic activity

The cells were culture in minimal medium and harvested when the OD_{600} was 4.0 and were washed with sterile water. Cell-free extracts were prepared as the crude enzyme in 100 $mmoll^{-1}$ Tris–HCl buffer (pH 7.5) with a proteinase inhibitor cocktail (for fungal/yeast cells; Sangon Biotech Co., Ltd., Shanghai, China) using a FastPrep cell homogenizer (Thermo Savant, Germany) as previously described

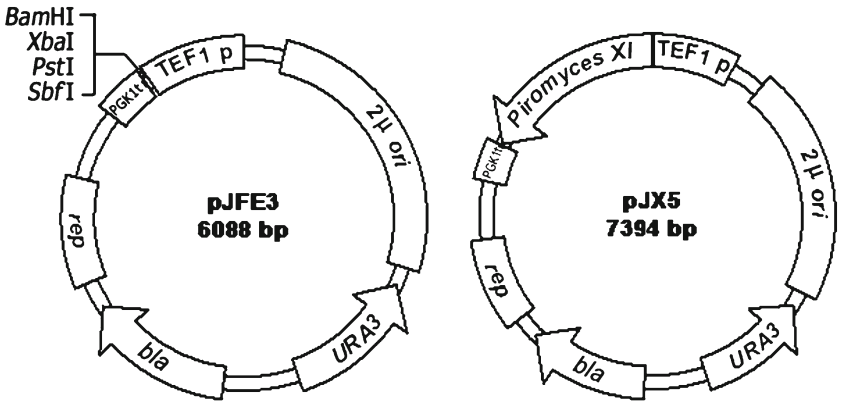

Fig. 1 Plasmids pJFE3 and pJX5

Table 1 Strains and plasmids used in this study

Saccharomyces cerevisiae strains and plasmids	Description	Sources
S. cerevisiae strains		
CEN.PK 113-5D	*MAT***a** *MAL2*-8^c *SUC2 ura3*-52	Entian and Kötter (1998)
BSPC042	CEN.PK 113-5D derivative; (−194, −1)*XKS1::loxP-TEF1p gre3*(−241, +338)*::TPI1p-RKI1-RKI1t-PGK1p-TAL1-TAL1t -FBA1p-TKL1-TKL1t-ADH1p-RPE1-RPE1t-loxP cox4::loxP-kanMX4-loxP/*(XK, *gre3*::PPP, and *cox4Δ*)	Peng et al. (2012)
BSPC095	BSPC042 derivative; {pJX5}*/*(*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*)	This work
BSPX013	Isolated after the adaptive evolution of BSPC095*/*(*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE)	This work
BSPC002	CEN.PK.113-5D derivative; (−194) *XKS1::loxP-TEF1p*	Peng et al. (2011)
BSPC081	Isogenic of BSPC002; *gre3*(−241, +338)::*TPI1p-RKI1-RKI1t-PGK1p-TAL1-TAL1t-FBA1p-TKL1-TKL1t-ADH1p-RPE1-RPE1t-loxP*	This work
BSPC016	Isogene of BSPC002; {pY7}/(XR, XDH, and XK)	This work
BSPC112	Isogene of BSPC002; {pJX5}*/*(*Pi*-XI and XK*)*	This work
BSPC212	Isogene of BSPC081; {pY7}/(XR, XDH, XK, *gre3*::PPP)	This work
BSPC312	Isogene of BSPC081; {pJX5}*/*(*Pi*-XI, XK, and *gre3*::PPP)	This work
Plasmids		
pY7	2 μ, containing *XYL1*, *XYL2* genes, optimal XR/XDH expression ratio for xylose fermentation	Walfridsson et al. (1997)
pYMIKP	Integration vector of multiple copies, *kanMX4*	Wang et al. (2004)
pJFE3	YEplac195 derivative; 2 μ, *Amp*^{r}, *URA3 TEF1p-PGK1t*	This work
pJX5	pJFE3 derivative; 2 μ, *Amp*^{r}, *URA3*, *TEF1p-Pi-xylA-PGK1t*	This work

(Peng et al. 2012). Protein concentration was measured using a BCA protein assay reagent kit SK3051 (Sangon Biotech Co., Ltd.). A spectrophotometer (Helios Gamma, Thermo Fisher Scientific, Waltham, MA) was employed to determine enzymatic activity by measuring the concentration change of reduced and oxidized forms of the coenzymes at 340 nm.

The xylose isomerase activity of the cell extracts was determined at 30 °C. The 1-ml reaction mixture contained 100 mmoll^{-1} Tris–HCl buffer (pH 7.5), 10 mmoll^{-1} $MgCl_2$, 500 mmoll^{-1} xylose, 1 U of sorbitol dehydrogenase (Roche, Boulder, CO), 0.15 mmoll^{-1} NADH, and 0.05 ml of the cell extract (Kersters-Hilderson et al. 1987). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of coenzyme/min, and the specific activity was expressed in units per milligram of protein.

The activity of xylose reductase and xylitol dehydrogenase in the cell extracts were measured at 30 °C using a previously described method (Eliasson et al. 2000b). One unit of enzyme activity was defined as the amount of enzyme required to reduce or oxidize 1 μmol of coenzyme/min, and the specific activity was expressed in units per milligram of protein.

Extracellular metabolite analysis and calculation

The concentrations of glucose, xylose, xylitol, glycerol, acetate, and ethanol were determined using HPLC using an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA) as previously described (Peng et al. 2012). The biomass concentration, the biomass yield, and the specific consumption or production rates for xylose, xylitol, glycerol, acetate, and ethanol, as well as carbon recovery, were calculated as previously described (Peng et al. 2012).

The biomass was determined by measuring the optical density of the culture at 600 nm using a spectrophotometer (Eppendorf AG, 22331 Hamburg, Germany) and the dry weight. Nitrocellulose filter membranes (0.45 nm; Sartorius, Göttingen, Germany) were pre-dried at 105 °C until a constant weight was achieved. Then, 5 mL of the cell culture was filtered and washed with three volumes of distilled water and dried at 105 °C. The weight differences of the

filter membranes with and without cells were determined as the dry weight of the biomass.

Gene expression profile analysis by microarray

The cells were cultured in minimal medium with 10 $g l^{-1}$ glucose and 20 $g l^{-1}$ xylose and were then collected during the exponential phase (OD_{600}=0.8) for RNA extraction. The total RNA was isolated using Trizol reagent (Takara, Tokyo, Japan) and purified using a NucleoSpin® Extract II Kit (Machery-Nagel Corp., Dueren, Germany). JINGXIN cDNA amplification and labeling kits (CapitalBio Corp., Beijing, China) and a two-channel 6K yeast genome array (CapitalBio Corp.) were employed for cy3 or cy5 labeling of the cDNA and microarray hybridization (Zhang et al. 2002). The array images were scanned using a LuxScan10KA Microarray Scanner (CapitalBio Corp.) and analyzed with LuxScan 3.0 software (CapitalBio Corp.). Space- and intensity-dependent normalization was employed based on a locally weighted scatter plot smoothing regression program (Yang et al. 2002). Fold changes in gene transcription ≥2 were considered significant, whereas changes ≥1.5 were also considered in the analysis. KegArray (http://www.genome.jp/kegg/expression/) was used to map the gene expression data to KEGG BRITE database for primary functional classification. The gene annotation information was based on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). For gene ontology (GO) analysis and pathway analysis, genes with changes in transcription level ≥2 were chosen, and the Molecule Annotation System (Küfer et al. 2001) (CapitalBio Corp.) was used.

Quantitative real-time PCR

The cDNA was reverse-transcribed from the mRNA using random oligonucleotides and a Reverse Transcription System kit (CapitalBio Corp.). qPCR analyses were performed using a SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) following the manufacturer's operation manual. *ACT1* was used as the normalization standard. All data were the average of triplicate values.

Plasmid copy number determination

Cells were cultured in minimal medium and harvested at OD_{600} 2.5. The TE buffer (10 mM Tris–Cl, pH 7.5, 1 mM EDTA) resuspended cells were broken by the FastPrep cell homogenizer (Thermo Savant, USA) as described above and the protein was removed by phenol chloroform method. The total DNA was precipitated by ethanol and resuspend by water. The total DNA was used to analyze the plasmid copy number by qPCR as described before (Lee et al. 2006). The specific primers (Table S1 in the ESM) for gene *Pi-xylA* exist in the plasmid only and the reference gene *ACT1* exist in yeast chromosome only were employed for the analysis. The amplification efficiency ratio of *Pi-xylA* and *ACT1* is close proximity to 1. The plasmid copy number was calculated according to the equation of plasmid copy number=$2^{CT(act1)}/2^{CT(xylA)}$.

Fluorescence microscopy

The cells were cultured until the late exponential phase in minimal medium containing 20 $g l^{-1}$glucose and 20 $g l^{-1}$ xylose at 30 °C. The cells were then fixed and stained with Calcofluor (Fluorescent Brightener 28, Sigma, St. Louis, MO) as previously described (Pringle 1991).

Results

Optimization of xylose metabolism via adaptive evolution

The recombinant plasmid pJX5 containing the *Pi-xylA* gene was introduced into the xylose downstream metabolic pathway-enhanced strain BSPC042 (XK, *gre3*::PPP, and *cox4Δ*), wherein xylulokinase and four enzymes (*RPE1*, *RKI1*, *TAL1*, and *TKL1*) of the nonoxidative pentose phosphate pathway were overexpressed, the aldose reductase gene *GRE3* was deleted and the electron transfer chain was disrupted by deleting the *COX4* gene (Peng et al. 2012). The resulting strain BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*) showed (0.47±0.00)$U mg^{-1}$ protein of XI activity, but no growth on xylose plates. However, weak growth was observed in liquid xylose minimal medium after 10 days of incubation.

Then, the adaptive evolution of BSPC095 was performed by sequentially transferring the culture into fresh xylose minimal medium during the early stationary phase. The cells showed gradually improved growth after more than 1,000 h of adaptive incubation on xylose. The doubling time was shortened from ~80 to 5 h (Fig. S1 in the ESM). The fastest growing colony of BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE) in xylose, with a μ_{max} of 0.11±0.00 h^{-1}, was selected. Although no base changes were found in the whole isomerase expression cassette (*TEF1p-Pi-xylA-PGK1t*) of the pJX5 plasmid recovered from the evolved BSPX013 strain, the XI activity in BSPX013 was surprisingly increased to 1.02 U±0.27 $U mg^{-1}$, which is approximately 2-fold higher than that of its parental strain BSPC095 (0.47 U±0.00 $U mg^{-1}$).

The results of the glucose–xylose cofermentation (Fig. 2; Table 2) revealed that the adaptive evolution process significantly increases the xylose fermentation capacity. The xylose-specific consumption rate of the evolved strain BSPX013 was 2.5-fold that of PC095 during glucose–

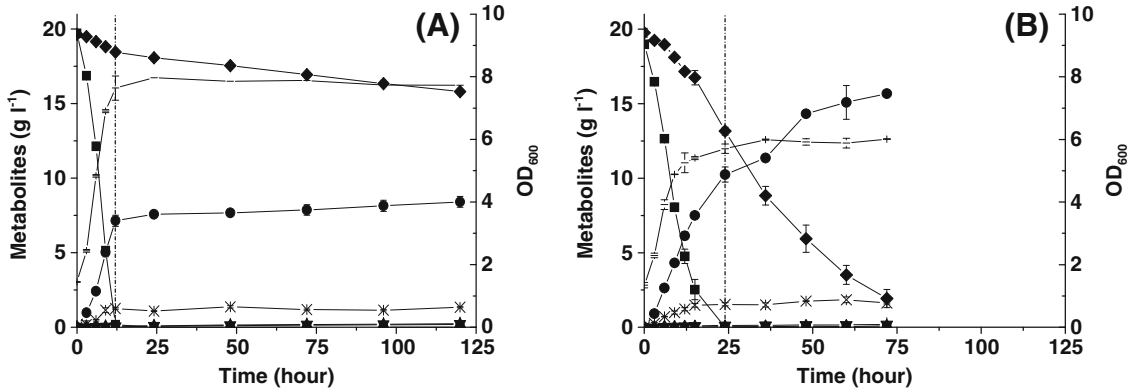

Fig. 2 Oxygen-limited shake flask fermentation of a glucose–xylose mixture by **a** BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*) and **b** the evolved strain BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE). *Black square*, glucose; *black diamond*, xylose; *black up-pointing triangle*, xylitol; *black circle*, ethanol; *error marks*, glycerol; and *solid line*, OD600. Fermentation was performed at 30 °C. Then, 20 gl^{-1} glucose and 20 gl^{-1} xylose were used as the carbon source. One unit at an OD_{600} was equal to 0.23 gl^{-1} of BSPC095 biomass and 0.26 gl^{-1} of BSPX013 biomass. The glucose–xylose cofermentation phase of BSPC095 and of BSPX013 lasted 12 and 24 h, respectively

xylose cofermentation phase and 8.5-fold that of BSPC095 during the xylose fermentation phase. The reference strain only utilized 3.68 gl^{-1} xylose within 120 h, whereas BSPX013 consumed 17.83 gl^{-1} xylose within 72 h. The consumed xylose was mainly converted into ethanol, and the ethanol yield was 0.43 gg^{-1} of consumed total sugar. However, the evolved strain required an additional 12 h to consume glucose completely (Fig. 2b).

The potential effect from plasmid change was studied. The plasmid copy numbers relative to the chromosomal gene *ACT1* in BSPC095 and BSPX013 are similar, which are 7.2±0.1 and 6.5±0.4, respectively. Moreover, the strain obtained by transforming the plasmid re-isolated from the evolved strain BSPX013 into strain BSPC042 did not show better growth on xylose than strain BSPC095, and no obvious difference on xylose metabolism were observed between the strain BSPX013 and its derivate strain, which were obtained by transforming the plasmid removed BSPX013 by original plasmid pJX5.

Table 2 Physiologic characteristics of the engineered *Saccharomyces cerevisiae* during batch fermentation

Strains	BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*)	BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE)
Fermentation time (h)	125	75
Consumed sugars (gl^{-1})		
Glucose	19.66±0.07	18.90±0.24
Xylose	3.68±0.23	17.83±0.31
Products yield (gg^{-1})		
Biomass[a]	0.076±0.005	0.064±0.001
Xylitol[b]	0.05±0.00	<0.01
Ethanol[c]	0.37±0.02	0.43±0.00
Specific substrate consumption rate during glucose–xylose cofermentation (g (g biomass)$^{-1}h^{-1}$)		
Glucose	1.50±0.02	1.07±0.03
Xylose	0.08±0.02	0.20±0.01
Specific substrate and products consumption or production rate during xylose-fermention phase after glucose depletion (g (g biomass)$^{-1}h^{-1}$)		
Xylose	0.02±0.000	0.17±0.000
Xylitol	0.001±0.000	0.001±0.000
Glycerol	0.001±0.000	0.002±0.001
Acetate	0.001±0.000	0.001±0.000
Ethanol	0.01±0.000	0.09±0.000
Carbon recovery	1.04±0.020	0.95±0.000

Values are given as the average and experimental deviation of two independent experiments

[a]Biomass yield was calculated based on glucose for the cultivation of glucose and xylose mixture, or based on xylose for the cultivation with xylose as sole carbon source

[b]Xylitol yield was calculated based on consumed xylose

[c]Ethanol yield was calculated based on consumed glucose and xylose

Transcriptional profile of adaptive evolution strain

To investigate the possible genetic factors that improved yeast xylose fermentation, the global transcription profile of the cells cultivated with glucose and xylose was analyzed. The data from the microarray were confirmed using qPCR (Table 3).

Compared with BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*), the evolved strain BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE) showed that 172 genes were upregulated by >1.5-fold and 59 genes by >2-fold, whereas 107 genes were downregulated by >1.5-fold and 45 genes by >2-fold. The GO analysis enriched proteins mainly involved in response to stress (GO:0006950), glycogen biosynthesis (GO:0005978), trehalose biosynthesis (GO:0005992), cellular response to nitrogen starvation (GO:0006995), nitrogen compound metabolism (GO:0006807), cell wall organization and biogenesis (GO:0007047), amino acid transport (GO:0006865), etc.

The transcription levels of most genes in the glycolysis and pentose phosphate pathway involved in xylose fermentation did not change significantly, except for genes encoding 6-phosphofructo-2-kinase (*PFK27*) and pyruvate decarboxylase isozyme (*PDC6*), which increased by 1.69- and 1.75-fold, respectively (Fig. 3). The expression of glucokinase genes, *HXK1* and *EMI1*, decreased by >2-fold. *GDH2* (which encodes NAD^+-dependent glutamate dehydrogenase) and *GLN1* (glutamine synthetase), which encoded proteins involved in glutamate synthesis, were upregulated by 12.5- and 1.89-fold, respectively. The trehalose synthetic genes *TSL1*, *TPS2*, and *TPS3* were downregulated (2.6-, 1.9-, and 1.9-fold, respectively) similar to the glycogen synthesis genes *GLG1*, *GSY1*, *GSY2*, and *GLC3,* which were downregulated 2.2-, 5.9-, 1.6-, and 2.2-fold, respectively. The gene encoding transcription factor Yak1p (serine-threonine protein kinase), which regulates trehalose and glycogen synthesis by activating Msn2p/Msn4p (Busti et al. 2010; Lee et al. 2008), was reduced by 1.96-fold. Additional information regarding the changes in gene expression is listed in Table S2 in the ESM.

The effect of several genes involved in xylose metabolism in different engineered strains

Seven interesting genes were selected basing transcriptional analysis to study their effect on xylose metabolism. The central carbon metabolism genes *PFK27* and *PDC6,* the oligopeptide transporter gene *OPT2*, and a cell wall-localized protein gene *YLR040C* were overexpressed in serial strains that contained various modified pathways. They are BSPC016 (XR, XDH, and XK), BSPC112 (*Pi*-XI and XK), BSPC212 (XR, XDH, XK, and *gre3*::PPP), BSPC312 (*Pi*-XI, XK, and *gre3*::PPP), BSPX095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*), and BSPX013 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*, AE). The overexpression of these genes was confirmed by RT-qPCR (Table 3). In addition, the alkaline phosphatase gene *PHO13,* the high-affinity inorganic phosphate transporter gene *PHO84*, and the cell wall mannoprotein gene *CWP1*, which relative transcriptional levels in the evolved strain BSPX013 were 1.25-, 0.27-, and 0.15-fold of those in BSPX095, were disrupted in the same strains mentioned above.

The growth characteristic of the engineered strains on the xylose plates is shown in Fig. 4. The deletion or overexpression of the genes did not restore the growth of the respiratory-deficient strain BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*) on xylose. The new genetic operation in the xylose metabolic pathways of the well-modified strains BSPC212 (XR, XDH, XK, and *gre3*::PPP), BSPC312 (*Pi*-XI, XK, and *gre3*::PPP), and BSPX013 (*Pi*-XI, XK, *gre3*::

Table 3 Fold changes in the mRNA expression of different genes by RT-qPCR

Genes	Relative transcriptional level			
	BSPX013 vs. BSPC095[a]	BSPX013 vs. BSPC095[b]	ES strains vs. BSPC016[c]	ES strains vs. BSPX013[d]
PFK27	1.69	2.34±0.26	23±5.2	35±12
PDC6	1.75	1.17±0.09	5±1.6	845±240
OPT2	11.87	12.9±2.9	74±18.5	50±28
YLR040C	3.17	1.54±0.23	1.62±0.16	1.75±0.26

Values are given as the average and standard deviation of three independent measurements

[a] Data from the microarray

[b] Data from the RT-qPCR

[c] Engineered strains, BSPC016 derivatives. In each row, ES refers to the strain that overexpressed the gene, which are listed in the first column of each row

[d] Engineered strains, BSPX013 derivatives. In each row, ES refers to the strain that overexpressed the gene, which are listed in the first column of each row

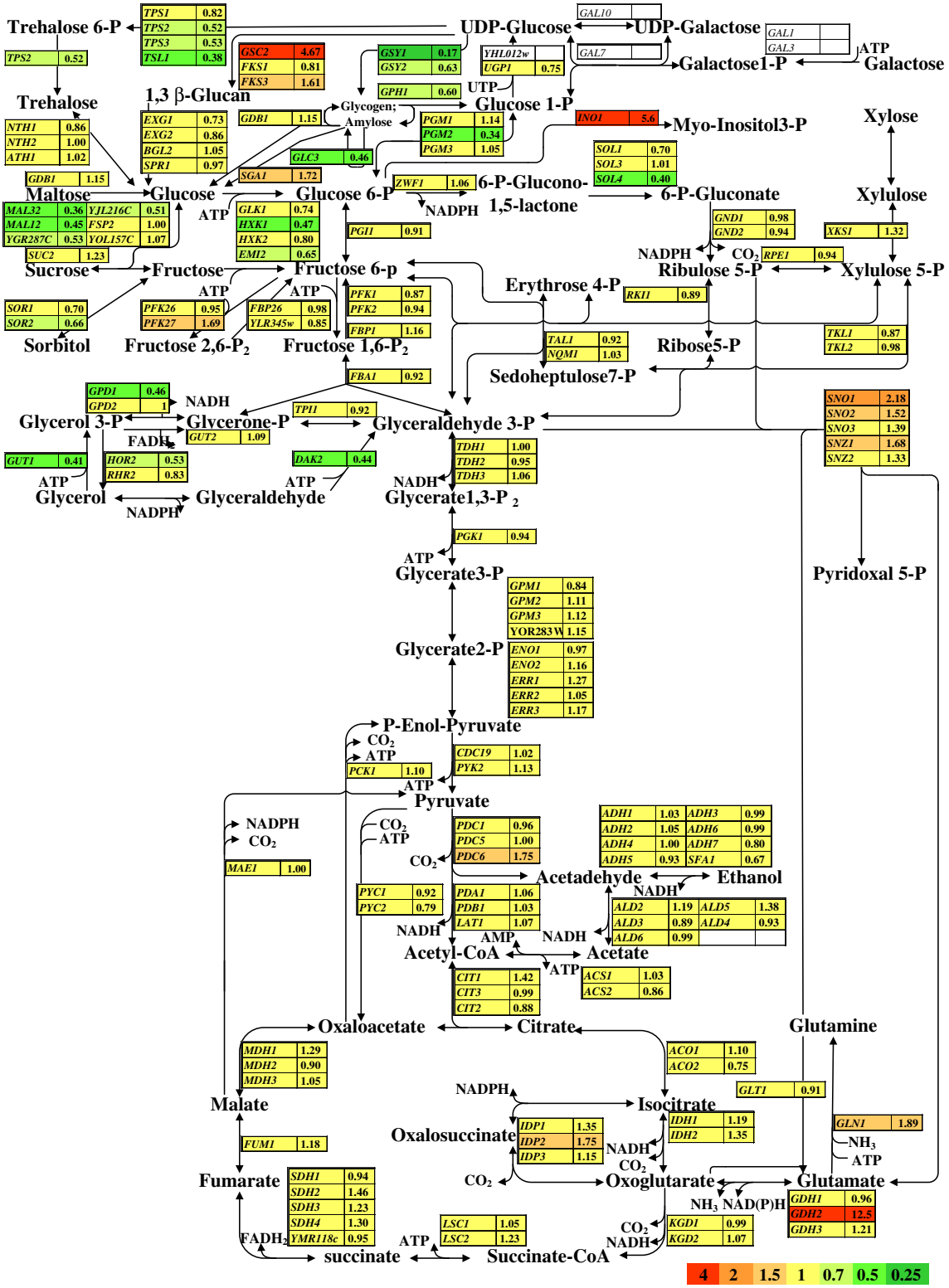

Fig. 3 Transcriptional changes in the genes involved in metabolic pathways were compared between the evolved strain BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE) and BSPC095 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, AE) did not further improve the growth of the strains on the xylose plates. The deletion of the *PHO84* gene arrested the growth of BSPC016 (XR, XDH, and XK) and BSPC112 (*Pi*-XI and XK) on xylose. This result reveals that

PPP, and *cox4Δ*). The cells were cultured in minimal medium with 10 $g l^{-1}$ glucose and 20 $g l^{-1}$ xylose and then collected during the exponential growth phase (OD_{600}=0.8)

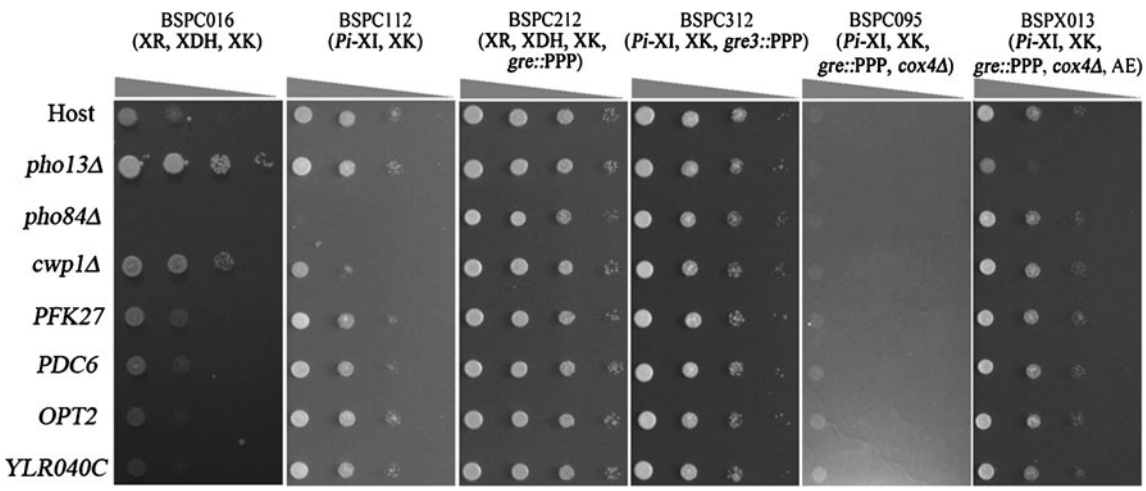

Fig. 4 The growth characteristics of the engineered strains on xylose plates. Samples of the serial 10-fold dilutions (4 μL) with an initial OD_{600} of 1 were spotted onto minimal medium plates with 20 gl^{-1} xylose as the sole carbon source and then cultured at 30 °C for 3 days

a functional Pho84p is necessary for xylose utilization of the strains contained only the xylose metabolic upstream pathways and metabolizes xylose inefficiently. In BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE), the expression of *PHO84* was downregulated by ~3.7-fold, and the deletion of *PHO84* gene did not affect the growth of strain on xylose. This suggested that the some unknown alteration compensated the function of Pho84p in BSPX013. *PHO13* deletion enhanced the growth of BSPC016 and BSPC112 on xylose plate, especially the XR-XDH background strain BSPC016. This result confirmed the earlier conclusions that deletion of *PHO13* gene can enhance the xylose utilizing of strains (Fujitomi et al. 2012; Van Vleet et al. 2008). However, deletion of *PHO13* gene in BSPX013, who can efficiently metabolize xylose, weakened the growth of that strain. Deletion of *CWP1* improved the growth of the XR-XDH background strain BSPC016. However, the opposite effect was observed in the *Pi*-XI background strain BSPC112. The other tested genes showed less effect on the cell growth. Hence, the effect of the tested genes on the different xylose-utilizing engineered strains was multifarious. Similar to the findings of Parachin et al. (2010), the effect was more significant in the strains that grew poorly on xylose. Therefore, the additional effects of these genes were further studied in the inefficient xylose metabolism strains. However, the results (Table S3 in the ESM) show that the BSPC112-derived (*Pi*-XI and XK) strains consumed too little xylose to evaluate. Hence, further discussion focused mainly on the BSPC016-derived (XR, XDH, and XK) strains.

Effect on the specific activity of XR and XDH

The specific activity of XR (with both cofactors NADPH and NADH) and XDH in BSPC016 (XR, XDH, and XK)-derived strains show that *PHO13* deletion and *PFK27* and *PDC6* overexpression slightly changes the enzymatic activity. The *CWP1* deletion decreased the enzymatic activity (Table 4).

Xylose aerobic fermentation of the strains

The fermentation data using xylose as sole carbon source under aerobic condition are shown in Table 5. Based on the BSPC016 (XR, XDH, and XK) strain, *PHO13* deletion positively affected the xylose utilization of the strain. The maximum specific growth rate (μ_{max}) of the strain increased from 0.039 to 0.168 h^{-1}. The xylose specific consumption rate increased from 0.156 to 0.203 gg^{-1} biomassh^{-1}. Then, 80 % xylose was consumed within 48 h by the *pho13Δ* strain, which produced ~1.6 gl^{-1} ethanol and less xylitol and glycerol accumulated compared with its reference strain BSPC016. These results demonstrate that inactivation of Pho13p improves ethanol production from xylose. Deletion of *CWP1* negatively affected the xylose utilization of the strain in the liquid culture, although the cells grew better on the xylose plate (Fig. 4). The μ_{max} of the *cwp1Δ* strain was 0.024 h^{-1}, which is lower than that of the control strain BSPC016 (0.039 h^{-1}). The *cwp1Δ* strain consumed only 3.69 gl^{-1} xylose during fermentation, and no metabolite was detected. The overexpression of *PFK27* and *PDC6* did not affect the μ_{max} of the strain in xylose. However, more biomass and less glycerol were produced by the *PDC6*-overexpressing strains. The glycerol production of the *PDC6*-overexpressing strain was only 24 % that of BSPC016.

Glucose–xylose cofermentation of the strains

The data for the glucose–xylose cofermentation under oxygen-limited conditions are listed in Table 6. Deletion of the *PHO13* gene did not affect the growth and consumption

Table 4 Activities of XR and XDH in the engineered *Saccharomyces cerevisiae*

S. cerevisiae strains	XR (U mg^{-1} protein)		XDH (U mg^{-1} protein)	XR/XDH ratio
	NADPH	NADH		
BSPC016	0.192±0.002	0.168±0.004	1.277±0.111	0.151
BSPC016/*pho13Δ*	0.216±0.002	0.181±0.003	1.513±0.085	0.143
BSPC016/*cwp1Δ*	0.040±0.004	0.043±0.002	0.614±0.088	0.066
BSPC016/*PFK27*	0.300±0.003	0.229±0.001	2.234±0.272	0.134
BSPC016/*PDC6*	0.251±0.017	0.214±0.001	1.900±0.029	0.132

Values are given as the average and standard deviation of three independent measurements

ability of the strains on glucose. However, during the xylose consumption phase (after glucose was exhausted), the *pho13Δ* strain showed higher xylitol and acetate production rates but lower glycerol and ethanol production rates, compared with the reference strain BSPC016 (XR, XDH, and XK). Deletion of *CWP1* decreased the xylose-metabolizing ability of the strain but increased their growth rate on glucose. The μ_{max} of the *cwp1Δ* strain was 35 % higher than that of the reference strain BSPC016 (0.383 vs. 0.284 h^{-1}) during glucose–xylose cofermentation. Therefore, the biomass and ethanol yield of the *cwp1Δ* strain were higher than those of BSPC016 (0.116 vs. 0.080 and 0.384 vs. 0.370 g g^{-1} total sugar). However, the lower specific consumption and production rate of the *cwp1Δ* strain during the xylose consumption phase indicates that deletion of *CWP1* decreases the xylose-metabolizing ability of the strain even though sufficient biomass was obtained from glucose. The overexpression of *PFK27* increased the specific production rate of acetate, which suggests that the acetaldehyde flux was increased. The *PDC6*-overexpressing strain consumed 14 % more xylose, both its ethanol and xylitol production rates increased and higher ethanol and xylitol yields were observed.

Deletion of *CWP1* affects the cell wall structure

Mannoproteins contribute to form the external cell wall layer, which determines wall porosity (Klis et al. 2006). One of the two major mannoproteins that localizes specifically to the birth scars of daughter cells (Smits et al. 2006) is encoded by *CWP1* (Zhang et al. 2008).

In the present work, deletion of *CWP1* led to the expansion of cells; the cells of the *cwp1Δ* strain were thus larger than its reference strain BSPC016 (XR, XDH, and XK) (Fig. S2A, B in the ESM). The cell dry weight of the *cwp1Δ* strain increased to 0.212 g l^{-1} OD^{-1}, whereas that of BSPC016 was 0.190 g l^{-1} OD^{-1}. Furthermore, the *cwp1Δ* strain was more readily stained with Calcofluor white (Fig. S2C, D in the ESM), which suggests that the cell wall structure of the strain was changed by the *CWP1* deletion.

At low xylose concentrations, such as at 4 g l^{-1}, xylose uptake is controlled at the transport level (Gardonyi et al. 2003; Runquist et al. 2009). The μ_{max} of the *cwp1Δ* strain in 4 g l^{-1} xylose was 0.008 h^{-1}, which is only 57 % that of BSPC016 (Fig. S3 in the ESM). The xylose uptake of the strain might have decreased after the deletion of *CWP1*.

Discussion

Glucose and xylose co-utilization is one of the most important economic strategies in the production of second-generation bioethanol using *S. cerevisiae*. However, the xylose metabolic regulatory mechanism needs to be understood further to enhance the xylose-fermenting efficiency of recombinant yeast strain (Hahn-Hägerdal et al. 2007; Jeffries and Jin 2004; van Maris et al. 2006).

In the present work, a strain was engineered via the expression of the xylose isomerase gene of *Piromyces* sp. (*Pi-xylA*), the overexpression of xylulokinase (*XKS1*) and the nonoxidative PPP genes *RPE1*, *RKI1*, *TAL1*, and *TKL1*, and deletion of the nonspecific aldose reductase gene (*GRE3*). However, the strain with the aforementioned genotypes did not exhibit sufficiently fast and effective xylose fermentation, similar to that previously reported in another study (Kuyper et al. 2005b). Then, the *COX4* gene that encodes subunit IV of cytochrome c oxidase was deleted to induce respiratory deficiency. The resulting strain BSPC095 (*Pi*-XI, XK, *gre3*::PPP, and *cox4Δ*) showed weakened growth on xylose. However, after prolonged adaptive evolution in xylose, which is an effective nonrational method of microorganism breeding, the evolved strain BSPX013 (*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, and AE) was obtained. BSPX013 showed a significantly improved capacity for fermenting xylose, and the fermentation was an anaerobic one since the respiratory was destroyed. The strain consumed 17.83 g l^{-1} xylose within 72 h; however, the glucose consumption rate was slightly decreased (Fig. 2). The increased enzymatic activity of xylose isomerase may have contributed to this improvement. However, no base change was found in the whole isomerase expression cassette (*TEF1p-Pi-xylA-PGK1t*), and the plasmid copy number was not increased after evolution. The mechanism underlying this unexplained increase needs to be elucidated.

Table 5 Metabolic characteristics of the engineered *Saccharomyces cerevisiae* in xylose aerobic fermentation

Strains	μ_{max}	Consumed xylose (gl^{-1})	Product yield (g (g consumed sugars) $^{-1}$)		Specific consumption or production rate[a] (mmol (g biomass)$^{-1}h^{-1}$)				
			Biomass	Xylitol	Xylose	Xylitol	Glycerol	Acetate	Ethanol
BSPC016	0.039±0.001	11.1±0.03	0.136±0.001	0.030±0.000	2.59±0.04	0.065±0.006	0.380±0.013	0±0.000	0±0.000
BSPC016/*pho13Δ*	0.168±0.002	22.6±0.21	0.137±0.005	0.013±0.001	2.46±0.08	0.030±0.001	0.008±0.000	0.049±0.005	0.277±0.028
BSPC016/*cwp1Δ*	0.024±0.001	3.69±0.01	0.267±0.002	0.000±0.000	0.97±0.06	0±0.000	0±0.000	0±0.000	0±0.000
BSPC016/*PFK27*	0.037±0.001	11.0±0.15	0.156±0.001	0.021±0.000	2.20±0.02	0.039±0.002	0.199±0.015	0±0.000	0±0.000
BSPC016/*PDC6*	0.037±0.000	12.2±0.28	0.160±0.003	0.034±0.001	2.36±0.01	0.089±0.002	0.216±0.003	0±0.000	0±0.000

Strains were cultured in the shake flasks for 144 h using 20 gl^{-1} xylose as sole carbon source. Values are given as the average and experimental deviation of two independent experiments

[a] Data of first 36 h

Table 6 Metabolic characteristics of the engineered *Saccharomyces cerevisiae* in glucose–xylose cofermentation under oxygen-limited conditions

Strains	Consumed glucose (gl^{-1})	Consumed xylose (gl^{-1})	Product yield (g (g consumed sugars)$^{-1}$)			Specific consumption or production rate[a] (mmol (g biomass)$^{-1}h^{-1}$)					Carbon recovery
			Biomass[b]	Xylitol[c]	Ethanol[d]	Xylose	Xylitol	Glycerol	Acetate	Ethanol	
BSPC016	19.40±0.16	10.25±0.53	0.080±0.001	0.109±0.011	0.373±0.012	0.494±0.027	0.064±0.008	0.023±0.003	0.015±0.002	0.422±0.041	0.91±0.03
BSPC016/*pho13Δ*	19.34±0.01	10.85±0.14	0.086±0.000	0.185±0.003	0.371±0.008	0.453±0.017	0.090±0.004	0.008±0.004	0.105±0.009	0.377±0.034	0.97±0.02
BSPC016/*cwp1Δ*	19.39±0.08	9.68±0.78	0.116±0.003	0.109±0.045	0.384±0.006	0.273±0.027	0.036±0.017	0.006±0.000	0.051±0.026	0.273±0.041	0.98±0.00
BSPC016/*PFK27*	19.30±0.01	10.59±0.17	0.085±0.001	0.099±0.009	0.383±0.000	0.433±0.009	0.052±0.006	0.007±0.000	0.087±0.012	0.452±0.011	0.95±0.00
BSPC016/*PDC6*	19.38±0.10	11.18±0.59	0.088±0.002	0.162±0.006	0.377±0.008	0.445±0.016	0.087±0.008	0.009±0.000	0.054±0.002	0.447±0.005	0.94±0.02

Strains were cultured in the shake flasks for 72 h using 20 gl^{-1} glucose and 20 gl^{-1} xylose as carbon source. A rubber stopper was used to maintain the oxygen-limited condition. The values are given as the average and experimental deviation of two independent experiments

[a] Specific consumption or production rates of metabolites were the data of xylose-consuming phase after glucose depletion

[b] Biomass yield was calculated based on consumed glucose only

[c] Xylitol yield was calculated based on consumed xylose only

[d] Ethanol yield was calculated based on consumed glucose and xylose

Then, the global transcriptional profile of the evolved strain was compared with the corresponding reference. The effect of several interesting genes was analyzed to investigate the mechanism for effective xylose fermentation.

The transport of xylose, which is regulated by hexose transporters in *S. cerevisiae*, is the first challenge in xylose utilization. In contrast to several previously reported evolved strain (Garcia Sanchez et al. 2010; Kuyper et al. 2005b), the expression of the hexose transporters (*SNF3*, *HXT1-7*, *HXT9*, *HXT11*, *HXT14*, and *HXT16*) was decreased at the transcriptional level after evolution. Except for the metabolic competition during increased xylose flux, the decreased transport capacity could account for the decrease in the glucose consumption rate.

Deletion of the cell wall mannoprotein gene *CWP1* reportedly produces cell wall abnormalities and enhances cell permeability in yeast (Zhang et al. 2008), which reduces the resistance of the yeast cells to metals and chemicals (dos Santos and Sá-Correia 2011; van Bakel et al. 2005). In the present work, after adaptive evolution, *CWP1* expression was downregulated by ~6.7-fold. The cells of the *CWP1*-deleted strain were bigger and heavier than those of the reference strain because of the changes in the structure and permeability of the cell wall. However, deletion of *CWP1* did not improve xylose utilization. It weakened the uptake of xylose, and decreased XR and XDH activity, which may account for the lower xylose utilization.

The *PFK27* and *PDC6* genes were upregulated by 1.69- and 1.75-fold, respectively, unlike most of the genes involved in glycolysis and the pentose phosphate pathway, which did not change significantly at the transcriptional level. *PFK27* encodes the isozyme of 6-phosphofructo-2-kinase, which catalyzes the synthesis of fructose-2,6-bisphosphate (F2,6bP) from fructose-6-phosphate and ATP (Boles et al. 1996; Kretschmer and Fraenkel 1991). In yeast, F2,6bP is the activator of 6-phosphofructo-l-kinase and inhibitor of fructose-1,6-bisphosphatase in vitro. It does not increase the glycolytic flux but rather is concerned with the homeostasis of metabolite concentrations (Müller et al. 1997). In this study, there was no obvious effect of *PFK27* overexpression to the ethanol production from glucose and xylose. *PDC1*, *PDC5*, and *PDC6* encode three different isozymes of pyruvate decarboxylase (Hohmann 1991). Kim et al. (2012) confirmed that reduced mRNA levels of pyruvate decarboxylase (*PDC1*) may cause low ethanol production from xylose. The specific rate of glycerol formation in the *pdcΔ* mutant was double that of the wild-type strain (Nevoigt and Stahl 1996). Pdc6p is not expressed during glucose fermentation but is induced during growth on nonfermentable carbon sources (Hohmann 1991). This finding indicates that increased *PDC6* expression may improve the metabolism of xylose, a nonfermentable carbon source (Jin et al. 2004). In this study, the positive effect of *PDC6* overexpression was demonstrated, and the *PDC6*-overexpressing strain consumed more xylose and produced less glycerol compared with the reference strain.

The expression of the genes *GDH2* and *GLN1*, which are involved in glutamate synthesis, were significantly upregulated in the evolved strain BSPX013. This result is consistent with that of a previous study that reported that *GDH2* and *GLN1* overexpression improves xylose utilization and ethanol production (Roca et al. 2003; Xiong et al. 2011). The increased *GDH2* and *GLN1* expression might contribute to the increased xylose fermentation in BSPX013.

Several "omics" studies have suggested a relationship between the stress response and xylose metabolism (Wenger et al. 2010; Wohlbach et al. 2011). In the evolved strain, the *YAK1* transcription level was reduced by 1.96-fold. *YAK1* encodes serine–threonine protein kinase, which phosphorylates and activates the transcriptional activators Msn2p/Msn4p (Lee et al. 2008). After adaptive evolution, lower Msn2p/Msn4p (~70 % of reference strain) expression levels were detected. Stress response genes, such as the trehalose and glycogen synthetic genes, were consequently downregulated. Trehalose and glycogen accumulate under carbon starvation, nitrogen limitation, and certain toxic stress conditions (Parrou et al. 1999). The downregulation of the trehalose and glycogen synthetic genes *TSL1*, *TPS2*, *TPS3*, and *GLC3* was also observed in xylose-cultured strain (Salusjärvi et al. 2006). In addition, the ethanol yield of *TPS1* and *TPS2* (trehalose 6-phosphate synthase genes) knockout *S. cerevisiae* strains from xylulose increased by 20 % (Eliasson et al. 2000a). The decrease in trehalose and glycogen synthesis might be another reason for the increased xylose utilization in the evolved strain.

Deletion of the *PHO13* gene, which encodes a protein that has both alkaline phosphatase activity specific for *p*-nitrophenyl phosphate and protein phosphatase activity, enhances the growth of *S. cerevisiae* on xylose and its ethanol production from xylose (Fujitomi et al. 2012; Van Vleet et al. 2008). Deletion of *PHO13* may alter the redox levels because increased acetate levels and decreased glycerol levels were observed during xylose fermentation (Van Vleet et al. 2008). This phenomenon was also confirmed by the results of the glucose and xylose fermentation (Tables 5 and 6) in this work. However, the transcription of *PHO13* was upregulated by 1.25-fold in the evolved strain BSPX013 and the deletion of *PHO13* in the evolved strain sharply weakened the growth of BSPX013 on xylose plate (Fig. 4). The most important differences of BSPX013 to other strains is the destroyed respiration. These results suggest that the positive effect of *PHO13* deletion to xylose fermentation might be depended on cellular respiration.

Using the adapted evolution strategy, the xylose fermentation ability of an engineered *Pi-xylA*-containing strain was significantly improved. The increased xylose isomerase

activity, the upregulated expression of genes involved in glycolysis and glutamate synthesis, as well as the downregulated expression of trehalose and glycogen synthetic genes might have contributed to the improvement.

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