J Ind Microbiol Biotechnol (2014) 41:27–39
DOI 10.1007/s10295-013-1350-y

BIOENERGY/BIOFUELS/BIOCHEMICALS

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Enhanced expression of genes involved in initial xylose metabolism and the oxidative pentose phosphate pathway in the improved xylose-utilizing *Saccharomyces cerevisiae* through evolutionary engineering

Jian Zha · Minghua Shen · Menglong Hu · Hao Song · Yingjin Yuan

Received: 21 April 2013 / Accepted: 17 September 2013 / Published online: 11 October 2013

Abstract Fermentation of xylose in lignocellulosic hydrolysates by *Saccharomyces cerevisiae* has been achieved through heterologous expression of the xylose reductase (XR)–xylitol dehydrogenase (XDH) pathway. However, the fermentation efficiency is far from the requirement for industrial application due to high yield of the byproduct xylitol, low ethanol yield, and low xylose consumption rate. Through evolutionary engineering, an improved xylose-utilizing strain SyBE005 was obtained with 78.3 % lower xylitol production and a 2.6-fold higher specific ethanol production rate than those of the parent strain SyBE004, which expressed an engineered $NADP^+$-preferring XDH. The transcriptional differences between SyBE005 and SyBE004 were investigated by quantitative RT-PCR. Genes including *XYL1*, *XYL2*, and *XKS1* in the initial xylose metabolic pathway showed the highest up-regulation in SyBE005. The increased expression of *XYL1* and *XYL2* correlated with enhanced enzymatic activities of XR and XDH. In addition, the expression level of *ZWF1* in the oxidative pentose phosphate pathway increased significantly in SyBE005, indicating an elevated demand for NADPH from XR. Genes involved in the TCA cycle (*LAT1, CIT1, CIT2, KGD1, KGD*, *SDH2*) and gluconeogenesis (*ICL1*, *PYC1*) were also up-regulated in SyBE005. Genomic analysis revealed that point mutations in transcriptional regulators *CYC8* and *PHD1* might be responsible for the altered expression. In addition, a mutation (Y89S) in *ZWF1* was identified which might improve NADPH production in SyBE005. Our results suggest that increasing the expression of *XYL1*, *XYL2, XKS1*, and enhancing NADPH supply are promising strategies to improve xylose fermentation in recombinant *S. cerevisiae*.

Keywords Xylose reductase · Xylitol dehydrogenase · Ethanol · Yeast · Evolutionary engineering

Introduction

Potential environmental and social benefits from the replacement of fossil fuels with bioethanol have driven the development of industrial *Saccharomyces cerevisiae* strains that can efficiently ferment all the hexoses (mainly glucose) and pentoses (mainly xylose) in lignocellulosic hydrolysates [1, 2]. Extensive metabolic engineering of *S. cerevisiae* has been performed towards that goal because natural *S. cerevisiae* cannot metabolize xylose [3–5]. The metabolic redesign often involves the reconstruction of a xylose catabolic pathway that converts xylose to xylulose through heterologous expression of the xylose reductase–xylitol dehydrogenase (XR–XDH) pathway or the xylose isomerase (XI) pathway [6]. Xylulose is then channeled into glycolysis after one step of phosphorylation and multiple biochemical reactions in the non-oxidative pentose

Electronic supplementary material The online version of this article (doi:10.1007/s10295-013-1350-y) contains supplementary material, which is available to authorized users.

J. Zha · M. Shen · M. Hu · H. Song · Y. Yuan (✉)
Key Laboratory of Systems Bioengineering, Tianjin University, Ministry of Education, Tianjin 300072, People's Republic of China
e-mail: yjyuan@tju.edu.cn

J. Zha · M. Shen · M. Hu · H. Song · Y. Yuan
Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China

H. Song
School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637457, Singapore

phosphate pathway (PPP). Although xylose consumption rates are much higher in XR–XDH-expressing strains than those in XI carrying strains, ethanol yields from xylose fermentation are much lower instead [7]. Therefore, more research attention is paid on the XR–XDH pathway for improved ethanol yields.

Low ethanol yields of XR–XDH-expressing strains are ascribed to formation of abundant byproduct xylitol due to intracellular cofactor imbalance, which is caused by poor recycling of redox cofactors in the initial oxidoreductive steps catalyzed by NADPH-preferring XR (encoded by *XYL1*) and strictly NAD^+-dependent XDH (encoded by *XYL2*) [4, 6]. Thus, many rational approaches to balancing cofactor recycling have been attempted. First, protein engineering of XR or XDH has been performed to alter the cofactor dependence for cofactor matching [8–10]. Second, regulation of cellular cofactor metabolism has been carried out to balance cofactor recycling for decreased xylitol formation and improved ethanol production [11–13]. However, these approaches do not always achieve efficient ethanol production from xylose, indicating that cofactor imbalance is not the only limitation in the XR–XDH pathway [14–17]. Approaches aimed at increasing metabolic flux or balancing the xylose pathway have also been applied to optimize xylose metabolism including overexpressing *XYL1* or *XYL2* [18–20], fine-tuning the expression of *XKS1* [21–23], balancing the expression of *XYL1*, *XYL2*, and *XKS1* [24], overexpressing genes in the non-oxidative PPP [25–27], and introducing xylose transporters [28, 29]. Although these genetic modifications have achieved some improvement in xylose fermentation, the best-performing strains exhibited lower ethanol production rate from xylose (0.13–0.45 g ethanol/g biomass/h) than that from glucose (1.2 g ethanol/g biomass/h) [15]. The underlying bottlenecks in the xylose pathway still need to be identified to accelerate ethanol production.

Evolutionary engineering has been widely used to improve the utilization of non-favored carbon sources such as arabinose, galactose, and lactose in *S. cerevisiae* [30–33]. It has also been successfully applied to improve xylose fermentation in recombinant *S. cerevisiae* [27, 32, 34]. This strategy complements rationally designed genetic modifications and reshapes the metabolic network in a global perspective [35, 36]. Moreover, the comparative analysis of the parent strain and the evolved strain can help to reveal the underlying mechanisms of evolution and facilitate the identification of potential targets that may be used to further improve the performance of yeast strains through inverse metabolic engineering.

In the present study, we genetically constructed a xylose-fermenting yeast *S. cerevisiae* SyBE004 expressing the XR from *Scheffersomyces stipitis* and a $NADP^+$-preferring XDH mutant [37]. Strain SyBE004 was then subjected to repeated adaptation using xylose as the sole carbon source, enabling the isolation of a mutant strain SyBE005 with improved xylose fermentation capability. To understand the molecular basis of such an improvement, comparative analysis of gene expression in chemostat culture of SyBE004 and SyBE005 was performed using real-time quantitative RT-PCR. This is the first report of the comparative study of the *S. cerevisiae* expressing $NADP^+$-preferring XDH. Genomic sequencing of both strains was applied to investigate the internal mechanism of the rearranged expression pattern in SyBE005. The results revealed several putative target genes related to the improved phenotype and will be useful for further metabolic engineering.

Materials and methods

Strains and media

The strains and plasmids used in the study are listed in Table 1. Yeasts were cultivated in YNB medium (6.7 g/l YNB, 20 g/l glucose, 2 g/l amino acid dropout) lacking appropriate amino acids. *E. coli* DH5α was used for regular cloning and was grown in LB medium supplemented with 100 mg/l ampicillin; 20 g/l agar was added in case of plate preparation. Oxygen-limited adaptive evolution was performed in YNB medium with 20 g/l xylose. Fermentation was conducted in YPX medium (10 g/l yeast extract, 20 g/l peptone, 20 or 50 g/l xylose) or YPGX medium (10 g/l yeast extract, 20 g/l peptone, 25 g/l glucose, 25 g/l xylose).

Yeast stock cultures were cultivated in YPX medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l xylose) at 30 °C in a rotary shaker of 200 rpm (Honour, Tianjin, China). Cells at stationary phase were harvested and stored in sterile glycerol solution (15 %, v/v) at −80 °C for future use.

Strain construction

The plasmids for overexpression of *PRE1*, *RKI1*, *TAL1*, and *TKL1* were constructed as follows. The individual expression cassettes, namely *TDH1p–RPE1–PGK1t*, *PGK1p–TAL1–PGK1t*, *PGK1p–RKI1–PGK1t*, and *TDH3p–TKL1–PGK1t* were obtained by fusion PCR of genomic DNA of yeast L2612 using the primers listed in Table S1. The expression cassettes *TDH1p–RPE1–PGK1t* and *PGK1p–TAL1–PGK1t* were then linked together by fusion PCR, digested by *Sac*I–*Xho*I and cloned into plasmid pAUR101 at the sites of *Sac*I and *Sal*I (Takara, Japan). Cassettes *PGK1p–RKI1–PGK1t* and *TDH3p–TKL1–PGK1t* were assembled similarly and cloned into plasmid pRS304 (ATCC) digested by *Xho*I and *Kpn*I.

The transformation was performed according to a previously reported method [38]. Prior to transformation,

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype	Sources
Strains		
L2612PR-D	D L2612PR, pRS305	[20]
SyBE004	L2612PR, *AUR1*:: pAUR101–RPE1–TAL1, *trp1*:: pRS304–RKI1–TKL1	This study
SyBE005	Evolved from SyBE004	This study
Plasmids		
pAUR101–RPE1–TAL1	pAUR101, *TDH1p–RPE1–PGK1t, PGK1p–TAL1–PGK1t*	This study
pRS304–RKI1–TKL1	pRS304, *PGK1p–RKI1–PGK1t, TDH3p–TKL1–PGK1t*	This study

plasmids were linearized by appropriate restriction enzymes. The transformants were selected in YNB plates with 20 g/l glucose lacking specific amino acids. Selection of the transformants carrying the plasmid pAUR101–RPE1–TAL1 was performed in YNB plate supplemented with 0.5 mg/l Aureobasidin A (Takara Bio, Kyoto, Japan). Correct transformation was confirmed by PCR amplification of gene cassettes from genomic DNA.

Fast DNA polymerase and DNA ligase used in this study were purchased from Transgene (Beijing, China). The restriction endonucleases were supplied by Takara (Kyoto, Japan). Kits for plasmid isolation and genome extraction were from Tiangen (Beijing, China). DNA fragments were separated on a 1 % agarose gel and purified with a gel extraction kit (Tiangen, Beijing, China). All the experiments were performed according to the manufacturers' instructions. DNA sequencing was completed by Beijing Genomic Institute (Beijing, China).

Evolutionary engineering on xylose

The evolution was carried out under oxygen-limited conditions in 50 ml of YNB medium (20 g/l xylose) in 250-ml flasks incubated in a rotary shaker (Honour, Tianjin, China) with a shaking speed of 150 rpm at 30 °C. Each batch of culture was initiated at a cell density of 0.2 (OD_{600}). Further cycles were repeated with the identical conditions and were ceased until the ultimate cell density became constant. After the evolution, cells from shake flasks were streaked on YNB plates with 20 g/l xylose to isolate single colonies. Twenty colonies with the biggest sizes were cultivated in YNB medium (20 g/l glucose) for 48 h, and then 100-μl culture of each colony was inoculated into 3-ml YNB medium (20 g/l xylose) in rubber-sealed test tubes and cultivated for 72 h (30 °C and 200 rpm). The cell density and metabolites were analyzed regularly after cultivation.

Aerobic growth and microaerobic fermentation

Yeast precultures were cultivated in YNB medium with 20 g/l glucose (30 °C and 200 rpm) and inoculated into fresh media when cells reached mid-log phase. Aerobic cell growth was carried out in 50 ml of YPX medium in 250-ml flasks with a starting cell density of 0.2 (OD_{600}) at 30 °C and 200 rpm. Microaerobic fermentation was performed in 100 ml of YPX or YPGX in 250-ml flasks with initial cell densities of 1.0 or 2.0 (OD_{600}). The flasks were sealed by rubber stoppers with needles and incubated at 30 °C and 150 rpm in a rotary shaker (Honour, Tianjin, China). Cell density was monitored by measuring the OD_{600} using a 756 spectrophotometer (Kanasi Inc., Tianjin, China). The experiments were repeated twice independently.

Chemostat cultivation

The inocula for chemostat cultivation were grown in YNB medium with 20 g/l glucose at 30 °C and 200 rpm. Cells at mid-log phase were inoculated into 0.3 l of YPX medium in a 0.7-l bioreactor (Bailun, Shanghai, China). The beginning cell density was 1.0 (OD_{600}). The continuous cultivation was conducted at 30 °C and 300 rpm, using YPX medium (20 g/l xylose) as the feed. The dilution rate was controlled at 0.05/h. The pH was maintained at 5.0 by addition of 1 M NaOH. Steady state was reached when the concentrations of biomass, xylose, and products remained constant for at least three consecutive volume changes.

Analysis of sugars and fermentation products

Sugars and fermentation products were analyzed on an HPLC system consisting of a Waters 1515 pump (Milford, MA, USA) and a Waters 2414 refractive index detector. The substrates were separated on an Aminex HPX-87H carbohydrate analysis column (Bio-Rad, Hercules, CA, USA) at 65 °C using 5 mM sulphuric acid as the mobile phase with a flow rate of 0.6 ml/min.

Enzyme assays

The cell samples at steady state from chemostat cultivation were harvested by centrifugation for 5 min at 4,000 rpm and 4 °C. The cells were washed twice with ice-cold water

and resuspended in the disintegration buffer [39]. Crude protein was prepared by 20-min sonication at 4 °C. Protein concentration was determined by using a Coomassie protein assay kit (Tiangen, Beijing, China). The activities of XR and XDH were measured by the method described previously [39]. One unit (U) of enzyme activity was defined as one micromole of coenzyme oxidized or reduced per minute, and the specific activity was defined as units per milligram of total protein.

Quantitative RT-PCR analysis

Samples at steady state of chemostat cultivation were harvested by centrifugation for 5 min at 4,000 rpm and 4 °C, followed by two washes with ice-cold water. Total RNA of cell samples was extracted by Mini RNA dropout kit (Tiandz Inc., Beijing, China). RNA integrity and quality were verified by RNA electrophoresis and NanoDrop-1000 (Thermo Scientific, Wilmington, USA). Totally 1 μg of RNA was used for each reverse transcription, which was performed at 37 °C for 1 h with the Reverse Transcription kit (Tiangen, Beijing, China). The cDNA products were then used for the quantitative PCR reaction by using the RealMaster Mix Kit (Tiangen, Beijing, China). For each reaction, a total volume of 20 μl was used consisting of 9 μl RealMaster Mix buffer, 0.5 μl each of forward and reverse primer (10 μM each), 0.5 μl cDNA template and 9.5 μl ddH_2O. The primers (Table S2) were designed according to the sequences in Saccharomyces Genome Database (http://www.yeastgenome.org). Quantitative RT-PCR was run in an ABI7300 Thermo cycler (Applied Biosystems, Carlsbad, CA, USA) and the conditions employed were as follows: 95 °C for 2 min; 94 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s (40 cycles). The threshold cycle value (C_t) for each sample was determined with the ABI7300 software. Three biological replicates were performed for each gene. The data was normalized using *ACT1* as the internal standard and analyzed according to the $2^{-\Delta\Delta CT}$ method [40]. Student's *t* test was used to statistically analyze the data using statistical function tools of Microsoft Excel.

Genomic analysis

The genomic sequencing and data analysis was commercially completed by GENEWIZ Inc. (Beijing, China). Genomic DNA of strains SyBE004 and SyBE005 was isolated to prepare sequencing libraries using Illumina's TruSeq sample preparation reagents. Illumina MiSeq system was used with sequencing cycles of 2 × 151 by MiSeq reagent kit v2 as a sequencing kit. The clean reads obtained after primary data analysis were mapped to reference genome (*S. cerevisiae* R64-1-1) in GenBank with BWA [41]. The results from the mapping were further used for calling of single-nucleotide polymorphisms (SNPs) and insertions and deletions (INDELS) in the reads compared with the reference sequence. Software SAMtools was used to determine the SNPs [42].

Results and discussion

Evolutionary engineering for improved xylose utilization

Batch fermentation of the genetically engineered strain SyBE004 was carried out in xylose medium to examine its xylose fermentation capability. The result showed that the xylose consumption rate of SyBE004 was only 0.260 g xylose/l/h (Table 2), which was far from the requirement of efficient xylose fermentation. In order to increase the xylose consumption rate, strain SyBE004 was subjected to repetitive adaptation in YNB medium with xylose being the sole carbon source. The process was monitored by measuring the growth rate, which was tightly coupled with the

Table 2 Summary of fermentation parameters of SyBE004 and SyBE005 under various conditions

	SyBE004	SyBE005	SyBE004	SyBE005	SyBE004	SyBE005
Carbon source	2 % (X)	2 % (X)	5 % (X)	5 % (X)	2.5 % (X) + 2.5 % (G)	2.5 % (X) + 2.5 % (G)
Q_{xyl} (g/l/h)[a]	0.260 ± 0.001	0.433 ± 0.000	0.473 ± 0.00	0.673 ± 0.010	0.358 ± 0.000	0.574 ± 0.000
Q_{eth} (g/l/h)[b]	0.052 ± 0.000	0.142 ± 0.001	0.116 ± 0.00	0.213 ± 0.006	0.228 ± 0.007[c]	0.424 ± 0.002[d]
Ethanol yield (g/g)	0.20 ± 0.01	0.33 ± 0.01	0.25 ± 0.00	0.33 ± 0.01	0.30 ± 0.01[c]	0.37 ± 0.00[d]
Xylitol yield (g/g)	0.42 ± 0.00	0.12 ± 0.01	0.46 ± 0.00	0.23 ± 0.00	0.48 ± 0.01[c]	0.23 ± 0.00[d]
Glycerol (g/g)	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.00[c]	0.02 ± 0.00[d]

X xylose, *G* glucose

[a] Average volumetric xylose consumption rate

[b] Average volumetric ethanol productivity

[c] The values were analyzed based on total sugars and determined from the fermentation in 62 h

[d] The values were calculated based on total sugars and determined from the fermentation in 44 h

xylose consumption rate. After seven cycles of adaptation, the cell growth showed an obvious increase and did not exhibit any improvement in further adaptation (Figure S1). Most of the selected strains exhibited similar xylose consumption rates. One strain that showed the highest ethanol yield and lowest xylitol yield was named SyBE005 (Figure S2). SyBE005 was stable and could retain the improved ability of xylose utilization even after five cycles of growth (~30 generations) in a medium without xylose (data not shown).

To obtain a satisfactory performance of xylose fermentation in recombinant yeast *S. cerevisiae*, labor-intensive and large-scale genetic modifications such as overexpression of genes in the xylose metabolic pathway are usually required [6, 28]. In contrast, evolutionary engineering, which is a spontaneous process driven by the demand for energy or carbon source, can save time in identifying target genes or pathways and provide the genetic basis for altered phenotypes in turn. Evolutionary engineering can globally optimize the cellular phenotypes and maximize the cellular functions under challenging conditions. It has been proven to be an effective method of improving the abilities of yeasts to utilize non-favored substrates such as xylose and arabinose [43]. In this study, we used growth-coupled adaptive evolution to increase the xylose consumption rate of strain SyBE004 and optimize its ethanol production from xylose. Because xylose uptake and ethanol production is closely related to the growth of SyBE004, the growth-coupled adaptive evolution can rapidly accelerate the xylose consumption, increase the ethanol yield, and decrease the xylitol yield (Figure S1). It has been reported that growth-coupled evolution can achieve a significant increase in the production rate and a reduction in byproduct formation [36]. In one previous study, the growth rate of a recombinant *S. cerevisiae* on xylose was elevated by eightfold after aerobic evolution of 500 h [32]. Similarly, the specific xylose consumption rate was increased by 66 % after only seven times of batch cultivation [27]. The study here highlighted the feasibility of evolutionary engineering in reconfiguration of xylose metabolism in yeast.

Aerobic growth on xylose

To compare the physiological behavior of the evolved strain SyBE005 and the parent strain SyBE004, their growth was monitored on YPX medium under aerobic conditions. As shown in Fig. 1, SyBE005 demonstrated much faster growth than SyBE004. The maximum growth rate of SyBE005 reached 0.165 ± 0.000 h^{-1}, which was 48.6 % higher than that of SyBE004 (0.111 ± 0.003 h^{-1}). In addition, SyBE005 showed around onefold higher biomass production than SyBE004 (Fig. 1). Metabolite analysis demonstrated that xylose was used up by both strains (data not shown). The difference in final cell densities might be ascribed to the different patterns of metabolic flux distribution in SyBE004 and SyBE005. Taken together, the comparison of aerobic growth in this study suggested that the intracellular xylose metabolism of SyBE005 was configurated to achieve faster xylose consumption.

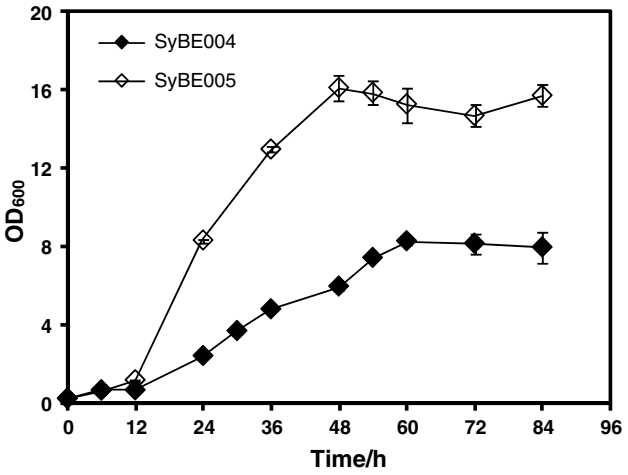

Fig. 1 Comparison of the aerobic growth of strains SyBE004 (*closed symbols*) and SyBE005 (*open symbols*) in 20 g/l xylose. The initial OD_{600} was 0.2 for each strain. Data points represent the average of duplicate independent culture

Microaerobic fermentation of xylose

To compare the xylose fermentation capabilities of SyBE004 and SyBE005, ethanol fermentation of both strains were investigated on 20 g/l xylose. Strain SyBE005 used up xylose in 48 h, and 6.80 g/l ethanol was produced with a yield of 0.33 g/g at the end of fermentation (Fig. 2a; Table 2). In contrast, strain SyBE004 consumed only 60 % of the xylose in 48 h, producing only 2.50 g/l ethanol with a yield of 0.20 g/g, which was 39.4 % lower than that in SyBE005 (Fig. 2a; Table 2). The xylitol yield in SyBE004 (0.42 g/g) was 2.5-fold higher than that in SyBE005 (0.12 g/g), which was consistent with the much higher ethanol yield in SyBE005. Glycerol formation in SyBE004 and SyBE005 was nearly the same (<0.3 g/l).

The performance of SyBE005 was also examined under a higher concentration of xylose (50 g/l) (Fig. 2b). To achieve a high ethanol productivity, the inoculum size was elevated to $OD_{600} = 2.0$ (Fig. 2b). Accordingly, the average xylose consumption rate approximately doubled in both strains compared with the fermentation in 20 g/l xylose, of which the inoculum size was 1.0 (OD_{600}) (Table 2). However, the xylitol yields increased by 10 % and 92 % to 0.46 and 0.23 g/g in strains SyBE004 and SyBE005, respectively, compared with the fermentation in 20 g/l xylose. This was consistent with a previous study where a higher

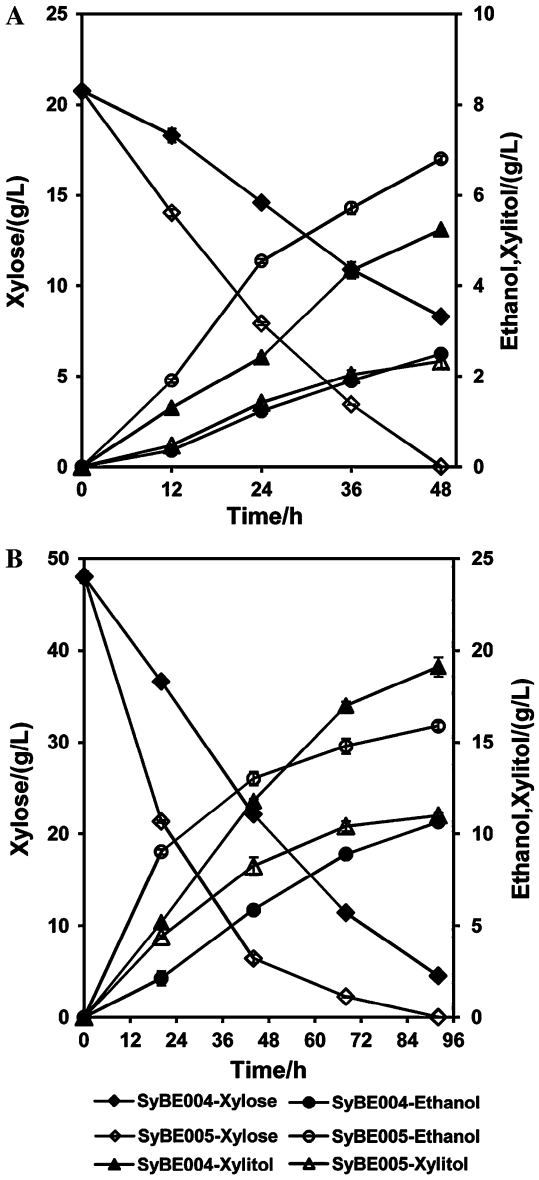

Fig. 2 Microaerobic xylose fermentation of strain SyBE004 (*closed symbols*) and strain SyBE005 (*open symbols*) in 20 g/l xylose and 50 g/l xylose. The initial cell density of inocula was adjusted to 1.0 of OD_{600}. The fermentations were carried out in 100 ml of media in 250-ml Erlenmeyer flasks. The data was the mean ± standard deviation of duplicated experiments

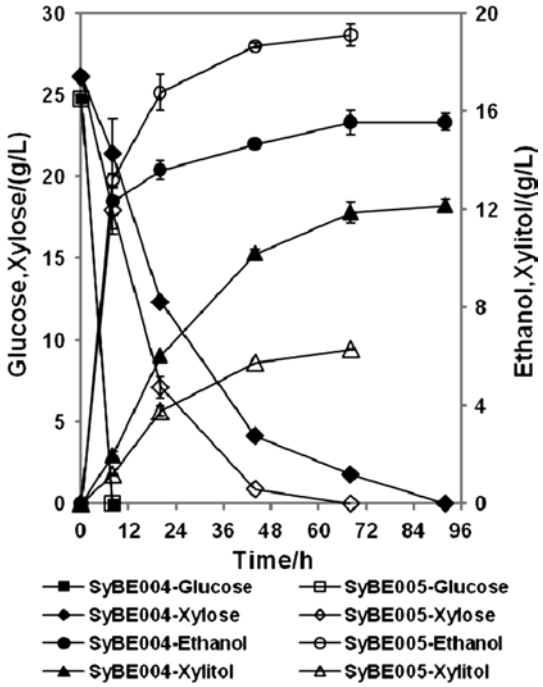

Fig. 3 Microaerobic fermentation of SyBE004 (*closed symbols*) and SyBE005 (*open symbols*) on mixed xylose and glucose, 25 g/l each. The initial cell density (OD at 600 nm) of inocula was adjusted to 2.0. The fermentation was performed in 100 ml of media in 250-ml Erlenmeyer flasks. The data was the mean ± standard deviation of duplicated experiments

initial cell density caused a higher xylitol production at the end of xylose fermentation [44]. In this study, the oxygen concentration reduced due to the increased inocula size in the cell culture, which was grown under microaerobic conditions in flasks. The cofactor imbalance was thus exacerbated, increasing xylitol production. However, the ethanol yields were unaffected by the increased xylitol production. The ethanol yields by SyBE004 and SyBE005 were 0.25 and 0.33 g/g, respectively. The average xylose uptake rate in SyBE005 was 42 % higher than that in SyBE004 (Table 2). Both strains produced similar amount of glycerol (Table 2).

Different evolutionary studies using nutrient limitation as selective pressure have highlighted the applicability of evolutionary engineering in optimizing constructed pathways related to cell growth [43, 45]. In this study, the adaptive evolution accelerated xylose consumption and increased ethanol yield simultaneously. The decreased xylitol yield might contribute to increased ethanol yield. One previous study showed that xylitol yield decreased by 55.7 %, accompanying a 22.5 % increase in ethanol yield through evolutionary engineering [27]. In another study, the xylose uptake rate increased by nearly twofold after 500 h of aerobic chemostat cultivation [46]. In summary, the results demonstrated that the xylose fermentation capability of strain SyBE004 was greatly improved through evolutionary engineering.

Co-fermentation of glucose and xylose

To achieve economical ethanol fermentation from lignocellulosic hydrolysates, efficient co-utilization of xylose and glucose is required. Thus, we evaluated the performance of SyBE004 and SyBE005 on mixed sugars (Fig. 3). Glucose was preferably utilized by both strains in 8 h, followed by

Table 3 Xylose consumption and products formation by SyBE004 and SyBE005 in chemostat cultivations at a dilution rate of 0.05/h

Strains	Xylose feed (g/l)	Xylose residual (g/l)	Xylose consumed (g/l)	Biomass (g/l)	q_{xylose} (mg/g biomass/h)	$q_{ethanol}$ (mg/g biomass/h)	$q_{xylitol}$ (mg/g biomass/h)
SyBE004	19.52 ± 0.14	18.32 ± 0.17	1.20 ± 0.03	0.87 ± 0.04	69 ± 1	24 ± 2	23 ± 1
SyBE005	19.28 ± 0.28	13.08 ± 0.80	6.20 ± 0.52*	1.39 ± 0.04*	223 ± 25*	88 ± 3*	5 ± 1*

The data were the mean ± standard deviation of triplicate samples

q_{xylose}, specific xylose consumption rate, represented as mg/g biomass/h; $q_{ethanol}$, specific ethanol production rate, represented as mg/g biomass/h; $q_{xylitol}$, specific xylitol production rate, represented as mg/g biomass/h

* Value is significantly different ($p < 0.01$) from SyBE004

Table 4 Enzymatic activities of XR and XDH from chemostat cultivations of SyBE004 and SyBE005

Strain	XR activity (U/mg protein)		XDH activity (U/mg protein)	
	NADPH	NADH	$NADP^+$	NAD^+
SyBE004	0.090 ± 0.005	0.111 ± 0.010	0.529 ± 0.020	0.091 ± 0.001
SyBE005	1.881 ± 0.136	0.452 ± 0.037	2.976 ± 0.227	0.321 ± 0.043

xylose consumption. SyBE005 utilized xylose much faster than SyBE004. After 44 h, about 97 % of xylose was consumed by SyBE005 and 18.67 g/l ethanol was formed with a yield of 0.37 g/g (Table 2). In contrast, SyBE004 only utilized 84 % of xylose, and the ethanol production reached 15.54 g/l with a yield of 0.30 g/g. The final xylitol yield for SyBE004 (0.42 g/g) was 109 % higher than that of SyBE005 (0.23 g/g). The results supported that the evolved strain SyBE005 had a superior capability of fermenting glucose–xylose mixture than that of SyBE004.

Chemostat cultivation of SyBE004 and SyBE005

Strains SyBE004 and SyBE005 were grown in fermenters by feeding YPX medium. At steady state, the specific xylose consumption rate and the specific ethanol production rate of SyBE005 was 2.2- and 2.6-fold higher than those of SyBE004, respectively (Table 3). The biomass concentration of SyBE005 was 59.8 % higher than that of SyBE004. Meanwhile, the specific xylitol production rate of SyBE005 was only 21.7 % of that in SyBE004.

The specific activities of XR and XDH of the chemostat cultures were measured and shown in Table 4. XR can utilize both NADPH and NADH as cofactors to catalyze the reduction of xylose. In SyBE004, the NADPH-dependent XR activity and the NADH-dependent activity were nearly equal while NADPH was preferred by XR in SyBE005. The total XR activity in SyBE005 was 11.60-fold higher than that in SyBE004. The XDH used here was a protein-engineered mutant that exhibited a reversed dependence of cofactors [37]. The mutant XDH uses $NADP^+$ or NAD^+ as a cofactor and prefers $NADP^+$ while native XDH strictly utilizes NAD^+ as the cofactor. The $NADP^+$-dependent XDH activity was much higher than the NAD^+-dependent XDH activity in SyBE004 and SyBE005. The total XDH activity in SyBE005 was 4.3-fold higher than that in SyBE004. Meanwhile, the ratio of $NADP^+$-dependent XDH activity to NAD^+-dependent XDH activity was higher in SyBE005, indicating enhanced preference of $NADP^+$ by XDH. The higher activities of XR and XDH in SyBE005 might contribute to the improved xylose consumption. Previous studies have reported high activities of XR and XDH are necessary for efficient xylose fermentation [19, 22, 47]. The great increase in XR and XDH activities can channel metabolism towards xylulose and downstream pathways more efficiently with an increased carbon flux, thus increasing xylose consumption while reducing xylitol production [17, 18].

Quantitative analysis of gene expression in SyBE004 and SyBE005

To determine the molecular basis of the improved xylose fermentation of SyBE005, quantitative RT-PCR was applied to measure the transcriptional changes of genes in initial xylose metabolism, glycolysis, PPP, and the TCA cycle. Cells were collected from chemostat cultivation instead of from batch cultivation, because in chemostat cultivation the environment is not changing and the dilution rate is defined, the growth rate and gene expression are constant.

All the genes that showed significant differences between SyBE004 and SyBE005 are listed in Fig. 4. Genes *XYL1*, *XYL2,* and *XKS1* showed the largest increase in expression levels among all tested genes in SyBE005 compared with SyBE004, ranging from 1.9- to 3.9-fold. Genes *ZWF1* and *RPE1* in PPP showed 63 % and 68 % enhanced expression in SyBE005, respectively. The genes in the upper glycolysis were mostly unchanged except *HXK1*, the

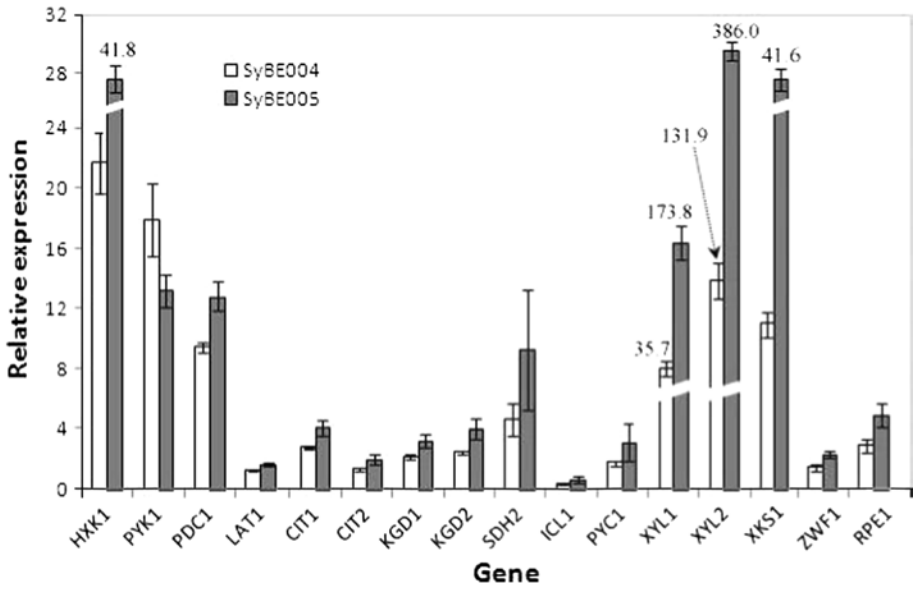

Fig. 4 Genes with significantly changed expression levels in strain SyBE005 compared with SyBE004. Chemostat cultivations of the strains were performed in 0.7-l bioreactors (300 rpm, pH 5.0) under anaerobic conditions at a dilution rate of 0.05/h. Cells at steady state were analyzed by real-time quantitative RT-PCR. The values were the ratios of expression levels of genes to internal standard *ACT1*. The data shown was the mean ± standard deviation of triplicate samples

expression of which was about onefold higher in SyBE005 than that in SyBE004. Up-regulated expression of *HXK1* is often observed when cells are grown on non-fermentative sugars [48]. The expression of *PYK1* decreased by 26 % in SyBE005 compared with SyBE004. Other genes that showed increased expression included genes in the TCA cycle (*LAT1, CIT1, CIT2, KGD1, KGD2, SDH2*) and gluconeogenesis (*ICL1*, *PYC1*). *PDC1* was the only gene in the fermentative pathway that displayed increased expression in SyBE005, which could relieve the Crabtree-negative effect and direct more carbon flux to the ethanol production pathway [49].

The enhanced expression of *XYL1* and *XYL2* might be related to the accelerated xylose consumption in SyBE005, which agreed well with the increased enzymatic activities of XR and XDH (Table 4). The increased activities of XR and XDH in the initial xylose metabolic pathway can result in a higher metabolic flux towards the non-oxidative PPP and glycolysis, which improves xylose fermentation. Previous studies have proven that high activities of XR and XDH are both required for efficient xylose utilization [47, 50]. The activity of XR partly determines the xylose consumption rate and has a great impact on product distribution [18, 20]. One study reported that integration of one extra copy of *XYL1* led to a 69 % higher xylose consumption rate and a 55 % lower xylitol yield [8]. Besides *XYL1*, the expression level of *XYL2* is also influential in xylose fermentation, especially in the product yields. A higher expression of *XYL2* results in a higher XDH activity, which facilitates an efficient conversion of xylitol into xylulose and avoids the accumulation of xylitol, leading to a lower xylitol yield and a higher ethanol yield [19, 47]. Another study also demonstrated that the 11.25-fold increase in XDH activity caused a 50 % reduction in xylitol formation [51]. Quantitative metabolomics analysis of SyBE004 and SyBE005 can provide the information of metabolic flux in initial xylose metabolic pathway and can enhance our understanding of the roles of increased activities of XR and XDH in improving xylose fermentation.

The genes of enhanced expression in the initial xylose metabolism also included *XKS1*, which has been considered as an overexpression target for improving xylose fermentation in many studies [21, 23]. Overexpression of *XKS1* doubled the ethanol yield and reduced the xylitol yield by 70 % in a recombinant xylose-fermenting *S. cerevisiae* [52]. Modeling-aided genetic modification of *XKS1* also confirmed the necessity of enough expression of *XKS1* to achieve an efficient xylose fermentation [53]. Integration of the *XKS1* gene from *E. coli* increased the growth rate by 54 % and decreased the xylitol yield by 89 % [53]. In this study, the ratios of the expression levels of *XKS1* to *XYL2* for both strains were nearly the same (1/9.14 and 1/9.28 for SyBE004 and SyBE005, respectively), indicating that the higher metabolic flux from xylulose from the first two steps might induce a higher expression of *XKS1* to keep the metabolism balanced. In one previous study, Du and coworkers [24] balanced the expression of genes (*XYL1*, *XYL2*, *XKS1*) in the initial xylose metabolism by a combinatorial transcriptional engineering method and achieved a 67 % higher ethanol yield and a 70 % faster xylose consumption. Thus, the activity of initial xylose metabolic pathway was enhanced to convert xylose into 5-phosphate-xylulose more efficiently in SyBE005 than in SyBE004, which could thereby reduce the accumulation of xylitol and accelerate xylose fermentation.

The comparative analysis also uncovered limiting steps in PPP. The 0.6-fold increase in the expression level of gene *ZWF1* might be a response to the elevated demand

for NADPH resultant from increased activity of XR which mainly uses NADPH as the cofactor (Table 4). The enzyme glucose-6-phosphate dehydrogenase encoded by *ZWF1* is a regulator in the oxidative PPP, which provides the majority of NADPH for cellular reactions [54]. Thus, the enhanced expression of *ZWF1* in SyBE005 would increase the supply of NADPH to meet the requirement of XR, whose activity was increased by 10.6-fold (Table 4). Increasing the expression level of *ZWF1* can increase xylose consumption rate at the expense of ethanol yield [12]. In another study, disruption of *ZWF1* decreased the xylitol yield by 83 % and increased the ethanol yield by 37 % [11]. The deletion of *ZWF1* reduced NADPH supply and compelled the use of NADH by XR, coupling with the conversion of xylitol by XDH which uses NAD^+ exclusively. The cofactor imbalance was therefore relieved to some extent, which facilitated the improvement in xylose fermentation. Differently, the XDH used here has a dual dependence on NAD^+ or $NADP^+$ and exhibits a preference on $NADP^+$, which can significantly relieve the cofactor imbalance. Thus, in this study the increased supply of NADPH could accelerate xylose consumption and would not increase xylitol formation as reported [12]. On the other hand, the enhanced demand for NADPH suggested that the balanced cofactor recycle of XR and XDH was not completely achieved although the cofactor dependence of XDH was reversed through protein engineering. In future studies, enhancement in *XYL2* expression might additionally balance the cofactor recycle and decrease the xylitol formation.

Another gene (*RPE1*) with increased expression was located in the non-oxidative PPP. Insufficient expression of genes in the non-oxidative PPP has been considered to be a reason for slow xylose metabolism [6, 15]. In previous studies, overexpression of genes in the non-oxidative PPP can improve xylose metabolism [26, 55]. In this study, stronger promoters *PGK1p* or *TDH3p* were applied to control PPP genes of *TKL1*, *TAL1*, and *RKI1* while a weaker promoter *TDH1p* was used to express *RPE1*, causing a relative low expression level. In xylose metabolism, the non-oxidative PPP works as the main catabolic pathway to metabolize xylulose converted from the initial metabolism pathway and channel flux towards glycolysis. On the other hand, it functions as the source to generate precursors for biosynthesis of aromatic amino acids and nucleic acids. Thus, it is quite important to maintain the balance between glycolysis and biosynthetic pathways of aromatic amino acids and nucleic acids when cells are grown on xylose [56]. Ribose-5-phosphate, an important precursor to synthesize aromatic amino acids, might be not enough to support the growth of SyBE004 on xylose. The increased expression of *RPE1* might result in a sufficient supply of ribose-5-phosphate that could be synthesized from ribulose-5-phosphate by the enzyme RKI1p. Thus, enhanced expression of *RPE1* in SyBE005 might help to support the growth of cells and keep the balance between glycolysis and biosynthetic pathways of aromatic amino acids and nucleic acids. Therefore, modulation of the expression of genes in the non-oxidative PPP is important; however, no such study has been reported. The result in this study stresses that fine-tuning the expression of genes such as *RPE1* might be necessary to achieve efficient xylose metabolism in recombinant *S. cerevisiae*.

The rearrangement also involved the TCA cycle. In this study, the significant up-regulation of genes in the TCA cycle showed that SyBE005 still metabolizes xylose in a respiratory pattern. The enhanced respiration might contribute to increased aerobic growth on xylose (Fig. 1). Previous studies have reported that xylose induces the expression of genes in the TCA cycle [49]. They were induced to a larger extent under oxygen-limited than fully aerobic conditions [49]. Moreover, a xylose-fermenting mutant carrying the oxoreductive pathway exhibited a physiological respiratory response without ethanol production and more than tenfold increase in expression of genes in the TCA cycle was observed [32]. Genes of the TCA cycle in combination with respiratory enzymes such as *NDI1* and *NDE1* can help to oxidize surplus NADH produced by XDH to maintain the balance of $NADH/NAD^+$ [32, 57]. Heterologous expression of a NADH oxidase from *Lactococcus lactis* decreased xylitol production and increased ethanol production by 70 % and 39 %, respectively [58]. The enhanced activity of respiration indicates that cofactor imbalance is still a problem in SyBE005 although the cofactor recycling has been balanced by the reversal of cofactor preference of XDH as mentioned above.

Besides reprogramming of the TCA cycle, significantly higher expression of genes *PYC1* and *ICL1* in gluconeogenesis was observed in SyBE005. The increased expression of these genes enhances the biosynthesis of 6-phosphate-glucose, which is the precursor to synthesize the glycan component of the yeast cell wall and other necessary components [59]. Enhanced gluconeogenesis has been observed as a common response to xylose in recombinant yeasts [32, 50, 59, 60]. However, direct evidence such as manipulation of the expression of genes in gluconeogenesis for the roles of these genes in xylose metabolism have not been reported.

Taken together, the comparative gene expression profiling suggested that the enhanced activity of initial xylose metabolism, increased NADPH supply, increased respiration and gluconeogenesis were related to the improved xylose utilization in strain SyBE005 (Fig. 5). In addition, increased expression of *RPE1* might also contribute to the improved xylose fermentation.

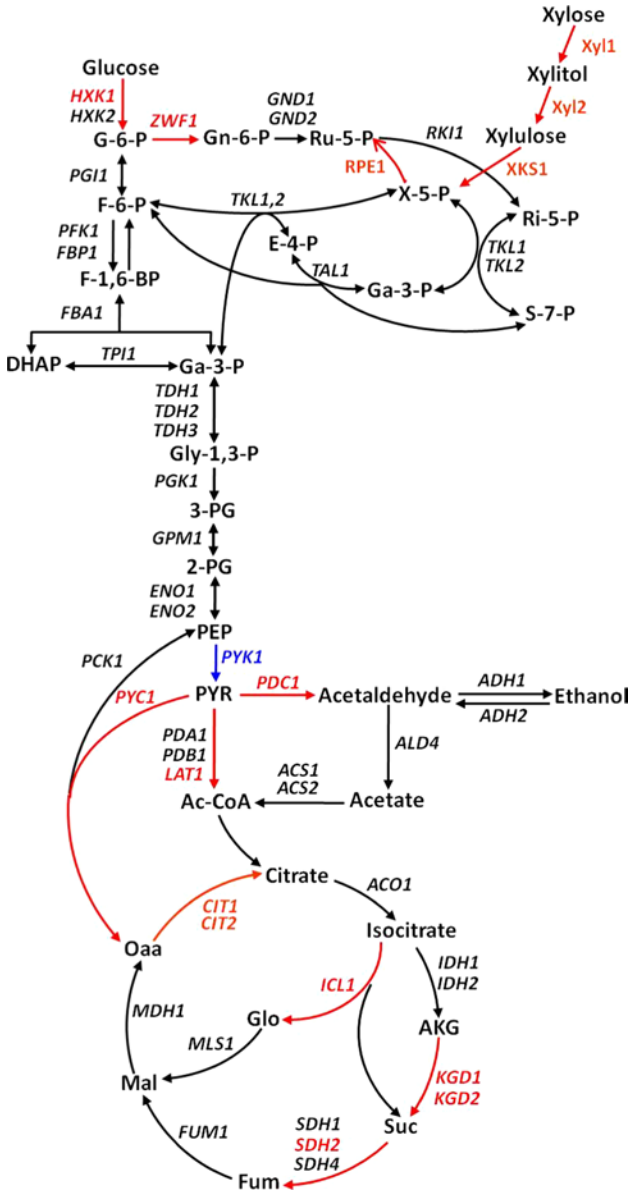

Fig. 5 Schematic illustration of transcriptional changes of genes in the xylose metabolic pathway and the central carbon metabolism pathway in strain SyBE005 compared with strain SyBE004. *Red arrowed lines* indicate significantly enhanced, and *blue* for depressed expression, reactions or pathways. *Black arrowed lines* indicate normal levels of expression, reactions or pathways

Genomic analysis of the evolved strain

The genome of the evolved strain SyBE005 was sequenced and compared with that of SyBE004, which identified 240 single-nucleotide polymorphisms (SNPs) in SyBE005. To determine the mutations responsible for changed expression levels of the genes investigated by RT-PCR, we focused on the SNPs that are related to carbon metabolism and regulation. Of the 240 SNPs, three were identified and assumed to be responsible for the changed expression of genes in SyBE005 (Table 5).

Table 5 The point mutations in the evolved strain SyBE005

Genes	Mutations	Functions
CYC8	$His^{537} \rightarrow Gln$	Tup1p–Cyc8p complex (regulation of glucose-repressed genes)
PHD1	$Glu^{261} \rightarrow Gly$	Transcriptional activator
ZWF1	$Tyr^{89} \rightarrow Ser$	The pentose phosphate pathway

One point mutation was found in gene *CYC8*, an important component of the Tup1p–Cyc8p complex [61]. The complex can interact with the transcriptional repressor Mig1p, which represses the transcription of genes whose expression is inhibited when glucose is present, such as those encoding enzymes for utilization of maltose, sucrose, or galactose [62, 63]. Xylose is a non-fermentable sugar and its metabolism is repressed when glucose is present. Hence, the mutation in *CYC8* might relieve the repression of the genes involved in the xylose metabolic pathway by Mig1p. In addition, it was observed that a point mutation occurred in the gene *PHD1* whose product can physically interact with the Tup1p–Cyc8p complex [64]. The coupled genomic mutation indicated that the Tup1p–Cyc8p complex might be the main factor for regulating the expression of genes in the metabolic pathway such as *XYL1* and *XYL2*. The detailed investigation of its role and the mutations will give insights into the mechanism of the interaction. Moreover, a mutation (Y89S) in *ZWF1* was identified (Table 5), which was consistent with the elevated expression of *ZWF1* in this study. The amino acid replacement might improve the activity of NADPH production to meet the requirement by xylose reductase.

In a word, the mutations in *CYC8* and *PHD1* after evolution might help to relieve the repression on transcription of genes in xylose metabolism. The mutation in *ZWF1* combined with its increased expression level increased the total activity of NADPH production to allow for efficient xylose utilization in SyBE005.

Conclusions

In this study, evolutionary engineering of a genetically engineered strain SyBE004 resulted in a mutant strain SyBE005 with a 2.6-fold higher specific ethanol production rate. The comparative RT-PCR analysis of SyBE004 and SyBE005 suggested that the evolution process involved upregulation of genes in the initial xylose metabolism, increased NADPH supply, increased expression of *RPE1*, and activated respiration and gluconeogenesis. Genomic analysis of SyBE004 and SyBE005 revealed that point

mutations in transcriptional regulators *CYC8* and *PHD1* might contribute to the increased expression. Moreover, a mutation (Y89S) in *ZWF1* was identified which might increase NADPH production for elevated demand by xylose reductase. The integrated analysis provides new targets for metabolic engineering of recombinant *S. cerevisiae* for efficient xylose fermentation.

Acknowledgments This work was funded by the National High Technology Research and Development Program ("863" Program: 2012AA02A701), the National Natural Science Foundation of China (Major International Joint Research Project: 21020102040), the National Basic Research Program of China ("973" Program: 2013CB733601), International Joint Research Project of Tianjin (11ZCGHHZ00500).

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