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ORIGINAL PAPER

A genetic overhaul of *Saccharomyces cerevisiae* 424A(LNH-ST) to improve xylose fermentation

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Abstract Robust microorganisms are necessary for economical bioethanol production. However, such organisms must be able to effectively ferment both hexose and pentose sugars present in lignocellulosic hydrolysate to ethanol. Wild type *Saccharomyces cerevisiae* can rapidly ferment hexose, but cannot ferment pentose sugars. Considerable efforts were made to genetically engineer *S. cerevisiae* to ferment xylose. Our genetically engineered *S cerevisiae* yeast, 424A(LNH-ST), expresses NADPH/NADH xylose reductase (XR) that prefer NADPH and NAD^+-dependent xylitol dehydrogenase (XD) from *Pichia stipitis*, and overexpresses endogenous xylulokinase (XK). This strain is able to ferment glucose and xylose, as well as other hexose sugars, to ethanol. However, the preference for different cofactors by XR and XD might lead to redox imbalance, xylitol excretion, and thus might reduce ethanol yield and productivity. In the present study, genes responsible for the conversion of xylose to xylulose with different cofactor specificity (1) XR from *N. crassa* (NADPH-dependent) and *C. parapsilosis* (NADH-dependent), and (2) mutant XD from *P. stipitis* (containing three mutations D207A/I208R/F209S) were overexpressed in wild type yeast. To increase the NADPH pool, the fungal GAPDH enzyme from *Kluyveromyces lactis* was overexpressed in the 424A(LNH-ST) strain. Four pentose phosphate pathway (PPP) genes, *TKL1, TAL1, RKI1* and *RPE1* from *S. cerevisiae*, were also overexpressed in 424A(LNH-ST). Overexpression of GAPDH lowered xylitol production by more than 40%. However, other strains carrying different combinations of XR and XD, as well as new strains containing the overexpressed PPP genes, did not yield any significant improvement in xylose fermentation.

Keywords Saccharomyces cerevisiae · Xylose fermentation · Ethanol · Redox balance · Pentose phosphate pathways

Introduction

Wild type *Saccharomyces cerevisiae* strains rapidly ferment glucose, mannose and galactose, but not xylose [1]. A great deal of effort has been made to generate strains of *S. cerevisiae* for fermentation of xylose by establishing the xylose utilization pathways from either fungi or bacteria [1–16]. Figure 1 schematically illustrates the initial metabolic pathways for xylose utilization in fungi. In *Pichia stipitis,* D-xylose is converted to D-xylulose by two oxidoreductases. First, xylose is reduced to xylitol by NADPH-dependent xylose reductase (XR; EC 1.1.1. 21 [17] and then xylitol is oxidized to xylulose by NAD^+-dependent xylitol dehydrogenase (XD; EC 1.1.1.9). Finally, xylulokinase (XK; EC 2.7.1.17) phosphorylates the xylulose into xylulose 5-phosphate, which is further metabolized through pentose phosphate pathway (PPP).

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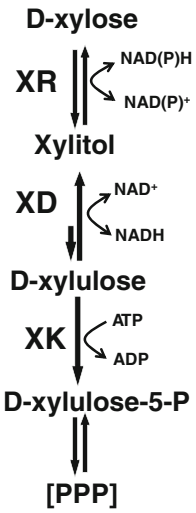

Fig. 1 The initial xylose utilization pathways in fungi. *XR* xylose reductase; *XD* xylitol dehydrogenase; *XK* xylulokinase; *PPP* Pentose phosphate pathway

We have constructed xylose fermenting *S. cerevisiae* by heterologous over-expression of *XYL1* and *XYL2* genes encoding XR and XD from *P. stipitis* (referred to as *Ps*XR and *Ps*XD, respectively) and *XKS1* gene encoding XK from *S. cerevisiae* (referred to as *Sc*XK) [10]. A stable recombinant *S. cerevisiae* strain, 424A(LNH-ST), was constructed by chromosomal integration of *Ps*XR, *Ps*XD and *Sc*XK genes. The *S. cerevisiae* 424A(LNH-ST) strain is capable of effectively fermenting four sugars (glucose, mannose, galactose, and xylose) presented in lignocellulosic biomass hydrolysates. However, the ethanol metabolic yield produced by this strain from xylose is about 75–85% of the theoretical maximum yield (unpublished results) and depends on the fermentation conditions. Productivity of ethanol from xylose is less than the productivity of ethanol from glucose. One explanation for lower ethanol yield could be production of xylitol as by product of xylose fermentation. Depending upon the xylose fermentation conditions, metabolic yield of xylitol from xylose range between 4 and 15% in *S. cerevisiae* 424A(LNH-ST) (unpublished data). This could be due to the difference in cofactor usage between the NADPH-dependent *Ps*XR and NAD^+-dependent *Ps*XD. Several strategies to reduce xylitol formation, which consist of modifying the affinity of the *Ps*XR and *Ps*XD enzymes towards more balanced cofactor utilization, were made [6, 10, 14–16, 18–20]. Some progress toward lower xylitol production during xylose fermentation was also made by changing the intracellular pool of NADPH [6]. Recently, over-expression of the genes involved in PPP metabolites has also been shown to improve xylose fermentation [21, 22].

In the present study, we have constructed four additional recombinant strains with different combinations of XR (from *P. stipitis*, *N. crassa* and *C. parapsilosis*), XD from *P. stipitis* (wild type and mutant, D207A/I208R/F209S) and the endogenous XK from *S. cerevisiae*. The fermentation of xylose was compared with our previous recombinant plasmid containing *Ps*XK-*Ps*XD-*Sc*XK. Xylose fermentation by our industrial strain 424A(LNH-ST) was also studied where (1) the NADPH pool was added by overexpressing fungal NADP-dependent GAPDH enzyme encoding by *GDP1* from *K. lactis* and (2) the flux through the PPP pathway was increased by overexpressing four PPP genes, *TKL1, TAL1, RKI1* and *RPE1* from *S. cerevisiae*.

Materials and methods

Strains and maintenance

The *S. cerevisiae* strains, 424A(LNH-ST), used in this work were described previously [4, 10, 23, 24]. *S. cerevisiae* strains were maintained on YEP medium, consisting of 10 g yeast extract and 20 g peptone per liter supplemented with glucose (20 g/l) [10] [YEPD] or xylose (20 g/l) [YEPX]. In all experiments, the antibiotic, hygromycin (Hyg) at a concentration of 125 μg/ml was present during growth and fermentation in the strain carrying Hyg-based plasmids. *Escherichia coli* Top10 competent cells (Invitrogen) were used as the host during development and characterization of various recombinant *E. coli* plasmids and yeast-*E. coli* shuttle plasmids.

Cloning of plasmids

Individual genes for *Ps*XR, *Ps*XD and *Sc*XK were cloned from pLNH plasmids described previously [10] using PCR. Primers used for PCR for these three genes are listed in Table 1. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The PCR fragments were digested by proper restriction enzymes (Table 1) and then cloned into pKS2μHyg [5, 10] and plasmid pKS2μHyg-*Ps*XD and pKS2μHyg-*Sc*XK (simply p*Ps*XD and p*Sc*XK) were generated. For XR cloning, first the PCR product was cloned into a TOPO vector (Invitrogen) to generate the pTOPO-*Ps*XRstop plasmid. The tail of xylulokinase gene (*XKS1*, Accession No. X61377) of *S. cerevisiae* was amplified from the genomic DNA using forward primer, D-F and reverse primer, D-R (Table 1). The template for the amplification of the gene was the genomic DNA of the strain 424A (LNH-ST) which was isolated using the DNA-Pure™ yeast genomic kit (PUREBiotech, LLC). The PCR fragment (*XhoI* and *SalI*) of XK-tail was cloned into the plasmid at the *SalI* site. Finally, this gene with promoter and tail regions was recloned into the pKS2μHyg plasmid between *BamHI* and *SalI* sites and the plasmid pKS2μHyg-*Ps*XR (simply p*Ps*XR) was generated.

Table 1 List of primers

No.	Name	Sequence
A-F	PYKpro-Xho-BamH-F	TGC**CTC GAGGGATCC**[a]GATCCAAATGTAAATAAACAATCACAAG
A-R	XDtail_Bgl-Sal-R	GAT**GTCGACAGATCT**TATAGTCGAAGGCTTTTCCGAATATTCG
B-R	XKtail_BamH-Sal-R	ATG**GTCGACGGATCC**GAGATGATTTAACAATAACCTAGC
C–F	ADC1pro_BamH-F	CCTCGAG**GGATCC**GGGATCGAAGAAATGATGGTAAATGAAATAG
C-R	XRtail_Xho-BamH-R	GCAT**GGATCCCTCGAG**TTAGACGAAGATAGGAATCTTGTC
D-F	XKtail-Xho-F	CATCATCAT**CTCGAG**GCGAACTGGAAAAGACTCTCATC
D-R	XKtail_Bgl-Sal-R	GTACCTAAG **GTCGACAGATCT**GAGATGATTTAACAATAACCTAGCTC
E-F	PGI1pro-BamH-F	ATCTCGAG **GGATCC**TCTGGGTGGGTGTGGGTG
F-R	PGI1pro-TKL1-R	CAATGTCAGTGAATTGAGT**CAT**[b]TTTTAGGCTGGTATCTTGATTCTAA
F–F	PGI1pro-TKL1-F	TTAGAATCAAGATACCAGCCTAAAA**ATG**ACTCAATTCACTGACATTG
G-F	PGI1-TAL1-F	CGATTTAGAATCAAGATACCAGCCTAAAA**ATG**TCTGAACCAGCTCAAAAGAAAC
G-R	PGI1-TAL1-R	GTTTCTTTTGAGCTG GTTCAGA**CAT**TTTTAGGCTGGTATCTTGATTCTAAATCG
H-R	TAL1-Sal-BamH-R	TT**GGATCCGTCGAC**AAATTAGTTCAGACTATAGATTAATGAGG
I–F	PGI1-Xho-F	TCTG**CTCGAG**TGTGGATCCATTGGATTATAGGAAGC
J-F	PGI1-RKI1-R	AACGCATCAATTTTTGGGACACCGGCAGC**CAT**TTTTAGGCTGGATCTTGATTCTAAATCG
J-R	PGI1-RKI1-F	CGATTTAGAATCAAGATCCAGCCTAAAA**ATG**GCTGCCGGTGTCCCAAAAATTGATGC
K-R	RKI1-Sal-R	GATGGAGAAT**GTCGAC**CTGCAGATCTGGGCAAACAAACAAG
L-F	PGK1-Xho-F	AACTATCGCATAC**CTCGAG**TTAGATCTGCCGATTTGGGCGC
M-R	PGK-RPE-R	ACTGGGAGCTATAATTGGTTTGAC**CAT**TGTTTTATATTTGTTGTAAAAAGTAG
M-F	PGK-RPE-F	ACTTTTTACAACAAATATAAAACA**ATG**GTCAAACCAATTATAGCTCCCAGTATCCTTG C
N-R	RPE-XhoI-R	CGAGGACACTT**CTCGAG**AAGGGATCCAAGAAAATCGAGAG

All primers are in 5′ to 3′ direction

[a] Sequences in boldface indicate restriction enzyme site

[b] Sequences in underline boldface indicate ATG start codon (or CAT in reverse primer)

The gene for XR from *Candida parapsilosis* (*Cp*XR, Accession No. AY193716) and *Neurospora crassa* (*NcXR*, Accession No. AY876382), XD with three mutations (with D207A/I208R/F209S) from *Pichia stipitis* (*Ps*XDm, Accession No. AF127801) and GAPDH from *Kluyveromyces lactis* (*Kc*GAPDH, Accession No. AJ430565) were synthesized CODA Genomics, Inc (Irvine, California, USA) with *BamHI* and *BglII* restriction cloning sites. All these genes have promoter of phosphoglucose isomerase (Accession number M37267) and XK-tail from *S. cerevisiae* and were cloned into pKS57 plasmids. Silent mutations were incorporated to remove the different restriction enzyme sites. Finally the genes were recloned into the pKS2μHyg vector.

To clone the transaldolase, (*TAL1*, Accession No. X15953), transketolase (*TKL1* Accession No. X73224), D-ribose-5-phosphate ketol-isomerase (RKI1) EC:5.3.1.6) and D-ribulose-5-phosphate 3-epimerase (RPE1) (EC:5.1.3.1) genes, a polymerase chain reaction (PCR) was used and the primers are listed in Table 1. Two different promoters were used to drive these genes; phosphoglucoisomerase gene (PGI1) (EC:5.3.1.9) promoter for *TAL1, TKL1* and *RKI1* genes and 3-phosphoglycerate kinase (PGK1) (EC: 2.7.2.3) gene promoter for *RPE1* gene. PGI-TAL1, PGI-TKL1, PGK-RPE1, and PGI-RKI1 constructs were cloned into pKS2μHyg giving rise to plasmids p*Sc*TAL1, p*Sc*TKL1, p*Sc*RPE1 and p*Sc*RKI1, respectively.

The three-enzyme-cassette plasmid (p*Ps*XR-*Ps*XD-*Sc*XK) was constructed by inserting the different restriction fragments using T4 DNA ligase into pKS2μHyg vectors. In briefly, first the p*Sc*XK was used as a vector (digest with *BamHI*) and then added to the *BamHI*−*BglII* insert of p*Ps*XD and generated the p*Ps*XD-p*Sc*XK. Then gene fragment of *Ps*XR from p*Ps*XR (digested by *BamHI* + *BglII*) was ligated into the p*Ps*XD-p*Sc*XK plasmid at the *BamHI* site and generated the p*Ps*XR-p*Ps*XD-p*Sc*XK. The internal *BglII* sites in the XR and XK were removed by silent mutation. Similarly other three-enzyme cassette with different XR, XD was made by ligation into p*Sc*XK. Each PCR fragment was purified by gel extraction kit (QIAGEN Inc). The sequence of the genes was checked by sequencing and restriction digestion. Similarly, the four-enzyme-cassette, p*Sc*TAL1-p*Sc*TKL1-p*Sc*RKI1-p*Sc*RPE1 (simply p*Sc*4PPP) was made

by inserting the different fragments using T4-ligase into pKS2μHyg vectors.

Transformation of plasmids

Transformation of *E. coli* with recombinant plasmids was performed by electroporation [25]. Transformation of the *S. cerevisiae* strains with the plasmids was also carried out by electroporation as described previously [26] with some minor modifications. Wild type *S. cerevisiae* (ATCC 4124) or xylose fermenting strain 424A(LNH-ST) was transformed with different plasmids. The fast-growing hygromycin resistant colonies were picked up and the presence of plasmid was confirmed by penicillinase assay [27] in case of 4124 strains and also by plasmid isolation. Then hygromycin resistance colonies were further screened by growth in xylose medium or by enzyme activity measurements.

Enzyme activities

We measured the XR and XD enzyme activities in the different recombinant yeast strains (Table 2). The expression of the different enzymes was verified by measuring the individual enzyme activities after expressing in the wild type *S. cerevisiae* (ATCC 4124) strain. The enzymatic activities of expressed enzymes were tested in the crude cell extracts. For enzyme assays, yeast cells (bearing with different plasmids) were cultured until the density was 450 KU in YEPD medium with hygromycin, and crude cells extract was prepared using Y-PER reagent (Pierce) 0.2 ml of the reagent with protease inhibitors (Sigma, Catalog # P8215) per 0.1 g cell. Activities of XR and XD enzymes were measured as described by Watanabe et al. [18]. One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 μmol $NAD(P)^+$ or NAD(P)H per min. Protein concentration was determined with Bio-Rad Protein assay reagents (Bio-Rad, Laboratories Inc, Hercules, California, USA). Crude extract from *S. cerevisiae* (ATCC 4124) strain with pKS2μHyg plasmid was used as a control.

Determination of cell growth

The cell density was measured by Klett Colorimeter (Bel-Art products, Pequannock, NJ USA) equipped with color filter KS-66 (Catalog # T370140066, Bel-Art products) and expressed in Klett units (KU). The grow experiments were done in a 50 ml YEPX medium in a 300 ml baffled Erlenmeyer flask equipped with a sidearm (Bellco). Inoculum was added until cell density reached 50 Klett units (dry weight 0.59 g/l) and cell growth was determined by measuring the Klett values at different times.

Determination of biomass dry weight

The culture sample (5 ml) was filtered with preweighed nitrocellulose filters (pore size 0.22 μM, Millipore). After the filtration of the broth, the cell mass was washed with deionized water, dried in 80°C oven for 5 h, and weighed. Duplicate determinations varied by less than 1%.

Fermentation

All the fermentation was done under micro-aerobic conditions, which has been described previously [10, 23]. All cultures were pre-grown in YEPX medium and then transferred into 100 ml of the YEPD medium with hygromycin in a 300 ml baffled Erlenmeyer flask equipped with side arm. The cultures were incubated in a shaker at 30°C at 200 rpm and grown aerobically overnight. The next day, cells were harvested by centrifugation (5,000×*g*, 5 min, at room temperature), and resuspended in 2 ml medium. Then cells were added to a fresh 100 ml YEPX medium containing 70 g/l xylose in 300 ml Erlenmeyer flask to final optical density of 400 KU (dry weight 4.76 g/l). One milliliter of the culture mixture was taken to serve as zero-time sample and then the top of flask was closed by a sponge-stopper and sealed with 4-layers of Saran Wrap to allow fermentation to be carried out under micro-aerobic conditions. At different time points, cell density was measured in the Klett instrument and dry mass was calculated from a standard curve. Cell density was expressed in dry weight in Table 3. One milliliter of the fermentation mixture was also taken for monitoring the fermentation. All 1 ml samples were centrifuged for 5 min at 15,000 rpm and the supernatant was collected into a fresh tube and stored at −20°C. All experiments on xylose fermentation were performed at least in duplicate.

Analysis of fermentation products

The xylose and the fermentation products (xylitol, ethanol, glycerol and acetic acid) were analyzed by HPLC using Waters Alliance 2695 HPLC system with Aminex® HPX-87H and/or HPX-87P 300 × 7.8 mm columns (Bio-Rad laboratories, California) equipped with Empower 2 (Waters Software). For ethanol yield calculation, the theoretical yield of ethanol is 0.51 g ethanol/g xylose [28] was used.

Results

Yeast growth on xylose medium under aerobic conditions

Presence of a functional xylose pathway in, *S. cerevisiae* 4124 strain over-expressing different combinations of XR

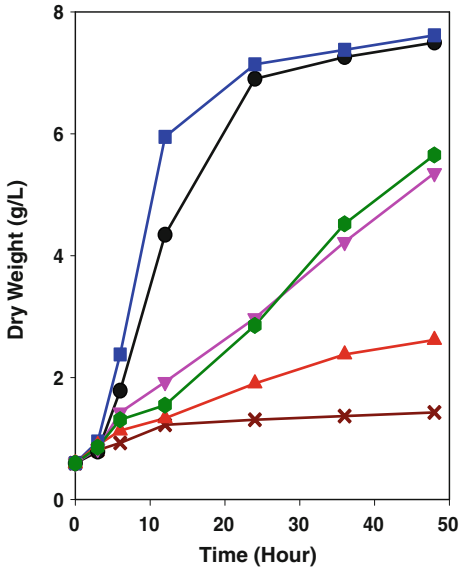

Fig. 2 Aerobic growth of new recombinant strains. YEP medium containing xylose (20 g/l) as the sole carbon source. Fresh culture was added in 50 ml medium in a 300-ml baffled Erlenmeyer flask until the cell density reached 50 Klett units (corresponding to dry weight 0.59 g/l). 4124 (*times*), 4124/p*Ps*XR-*Ps*XD-*Sc*XK (*filled circle*), 4124/p*Nc*XR-*Ps*XD-*Sc*XK (*filled square*), 4124/p*Cp*XR-*Ps*XD-*Sc*XK (*filled triangle*), 4124/p*Ps*XR-*Ps*XDm-*Sc*XK (*filled inverted triangle*) and 4124/p*Nc*XR-*Ps*XDm-*Sc*XK (*filled hexagon*) strains. The figure illustrates one representative of duplicate experiments for each strain, and duplicate Klett units varied by less than 2%

and XD was demonstrated by aerobic growth on xylose alone. Four new recombinant strains were compared for aerobic growth in 50-ml shaker-flask cultures with 20 g/l xylose. All of these strains were carrying a plasmid with three over-expressed genes (XR, XD and *Sc*XK). The wild-type *S. cerevisiae* 4124 strain (a negative control) and strain 4124/p*Ps*XR-*Ps*XD-*Sc*XK (a positive control) were used for comparison. Strain 4124/p*Nc*XR-*Ps*XD-*Sc*XK (where the XR gene was from *N. crasa*) grew aerobically on xylose similarly to the positive control strain (4124/p*Ps*XR-*Ps*XD-*Sc*XK) (Fig. 2). Slower growth was observed in the case of strains carrying the *Ps*XDm (3-mutation on *Ps*XD, with D207A/I208R/F209S) gene and very little growth occurred in the strain carrying *Cp*XR (where the XR gene was from *C. parapsilosis*). The negative control wild-type strain 4124 did not grow in xylose medium (Fig. 2).

Fermentation of xylose by different XR-XD-XK cassettes

Since all recombinant strains were able to grow on xylose medium, the fermentation of xylose under micro-aerobic conditions was tested. The fermentation of xylose by the different recombinant strains is shown in Fig. 3. Xylose consumption (Fig. 3a) and production of xylitol, glycerol, acetic acid and ethanol were monitored during fermentation (Fig. 3b; Table 3). In most cases no detectable amount of acetic acid was observed. The cell density reached 500–600 Klett units (correspond to dry weight 5.95–7.14 g/l) depending upon the strain. More than 95% of the xylose was consumed within 24 h in the strains carrying the plasmid p*Ps*XR-*Ps*XD-*Sc*XK and p*Nc*XR-*Ps*XD-*Sc*XK. Xylitol was produced during fermentation and the metabolic yield of xylitol was between 7 and 9% by these two strains (Table 3). The amount of ethanol produced by the strain carrying *Nc*XR was about 28 g/l, corresponding to a metabolic yield of 78.6% of theoretical. The ethanol metabolic yield by the control stain was 76.5% of theoretical. The metabolic yield of glycerol was 6.7 and 6.2% of theoretical in the strain carrying *Nc*XR and control strain, respectively. No detectable acetic acid was observed in these two strains.

The strain carrying *Cp*XR, in which the XR is mostly NADH-dependent, the consumption of xylose is very slow and only 15% of xylose was consumed within 48 h. Metabolic yield of xylitol was about 6% of the theoretical yield and the metabolic yield of ethanol was only 34%. Decrease in xylose consumption, increase in xylitol production and decreases in ethanol yield were observed in the strains carrying *Ps*XDm genes as compared to our control strain (with *Ps*XD). Decreases in xylitol production and increases in the ethanol yield were achieved by replacing the *Ps*XR by *Nc*XR using the same *Ps*XDm. But overall ethanol yield by the strain 4124/p*Nc*XR-*Ps*XDm-*Sc*XK was lower compare to our control strain 4124/p*Ps*XR-*Ps*XD-*Sc*XK. Among the four recombinant strains, only the strain carrying plasmid p*Nc*XR-*Ps*XD-ScXK had better ethanol yield than our control strain 4124/p*Ps*XR-*Ps*XD-*Sc*XK.

Enzymatic activities of XR and XD

The specific activities of the enzyme XR and XD in the recombinant *S. cerevisiae* strains are summarized in Table 2. The expressed activities of *Ps*XR and *Nc*XR were the same when NADPH was used as the co-factor in the assay. The measured activity of *Nc*XR was nearly an order of magnitude lower when NADH was used as the cofactor. The activity of *Ps*XR was only slightly lower when NADH was used as the cofactor. While the measured activity of *Cp*XR was higher when NADH was the cofactor, the total activity when NADH was used was significantly lower than *Ps*XR.

For *Ps*XDm, the activity using NAD^+ activity was ten times lower and the $NADP^+$ activity was four times higher than with *Ps*XD.

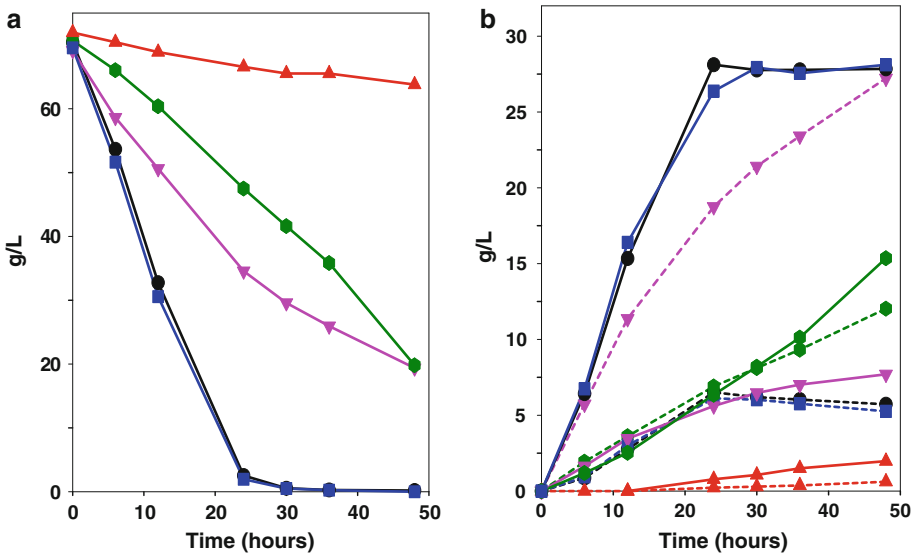

Fig. 3 Xylose consumption and products formation during fermentation by 4124 strains. The (100 ml) YEPX medium contained xylose (70 g/l). Xylose consumption (**a**) and ethanol production (**b**) are shown in *solid lines* and xylitol production (**b**) are shown in *dashed lines*. 4124/p*Ps*XR-*Ps*XD-*Sc*XK (*filled circle*), 4124/p*Nc*XR-*Ps*XD-*Sc*XK (*filled square*), 4124/p*Cp*XR-*Ps*XD-*Sc*XK (*filled triangle*), 4124/p*Ps*XR-*Ps*XDm-*Sc*XK (*filled inverted triangle*) and 4124/p*Nc*XR-*Ps*XDm-*Sc*XK (*filled hexagon*) strains. The figure illustrates one representative of duplicate experiments for each strain

Improvement of xylose fermentation by others genes

Slightly faster xylose consumption was observed in 424A (LNH-ST)/p*Kl*GAPDH and 424A(LNH-ST)/p*Sc*4PPP (Fig. 4; Table 3) as compared with our control strain 424A(LNH-ST), where three xylose metabolic genes (*Ps*XR, *Ps*XD, and *Sc*XK) were integrated into the chromosome. Xylitol production was reduced 42 and 23%, respectively, in 424A(LNH-ST)/p*Kl*GAPDH and in 424A(LNH-ST)/p*Sc*4PPP strains, as compared to the control strain (Table 3). Small improvements were observed in strains carrying GAPDH and 4PPP enzymes.

Discussion

Previously, we have developed a *S. cerevisiae* strain, 424A(LNH-ST), which can efficiently co-ferment both xylose and glucose [4, 10, 11]. *S. cerevisiae* strain, 424A(LNH-ST) consumes glucose faster than xylose and the rate of xylose utilization by *S. cerevisiae* 424A(LNH-ST) is 4–5 times lower than that of glucose [4, 10, 11] and xylitol metabolic yield was about 10% of the potential yield from xylose (Table 3). The production of xylitol has been mainly ascribed to imbalanced redox cofactors. The difference in cofactor preference of *Ps*XR (NADPH-dependent) and *Ps*XD (NAD^+-dependent) leads to the formation of xylitol under micro-aerobic fermentation conditions (Fig. 1). Therefore, xylitol is a byproduct in ethanol fermentation from xylose and its production reduces the final yield of ethanol [29]. To reduce the xylitol excretion during xylose fermentation, we constructed several *S. cerevisiae* strains (1) carrying different combinations of xylose reductase and xylitol dehydrogenase to balance the cofactor (Tables 2, 4), (2) increasing the NADPH pool by over-expressing the $NADP^+$-dependent GAPDH (3) increasing the flux through PPP pathway downstream of xylulose-5-phosphate by over-expression of the endogenous genes encoding enzymes from non-oxidative PPP (*TAL1*, *TKL1*, *RKI1* and *RPE1*).

P. stipitis xylose reductase has a higher specific activity for NADPH while xylitol dehydrogenase is NAD^+ specific (Table 4). The mode of xylose fermentation is incompatible under anaerobic conditions since it leads to a lower pool of NADPH and net production of NADH in the overall reaction [29]. There is no transhydrogenase in

Table 2 Specific activity of XR and XD in recombinant yeast strains

Strains with plasmid	XR (U mg^{-1})		XD (U mg^{-1})	
	NADPH	NADH	NAD^+	$NADP^+$
p*Ps*XR-*Ps*XD-*Sc*XK	0.19 ± 0.01	0.13 ± 0.01	4.63 ± 0.80	0.09 ± 0.01
p*Nc*XR-*Ps*XD-*Sc*XK	0.14 ± 0.01	0.02 ± 0	4.54 ± 1.06	0.10 ± 0.02
p*Cp*XR-*Ps*XD-*Sc*XK	0.01 ± 0	0.05 ± 0.01	3.11 ± 0.62	0.10 ± 0.01
p*Ps*XR-*Ps*XDm-*Sc*XK	0.20 ± 0.02	0.15 ± 0.02	0.39 ± 0.02	0.43 ± 0.03
p*Nc*XR-*Ps*XDm-*Sc*XK	0.14 ± 0.02	0.02 ± 0	0.46 ± 0.10	0.44 ± 0.02

XR xylose reductase, *XD* xylitol dehydrogenase, *XDm* mutant (D207A/I208R/F209S) xylitol dehydrogenase

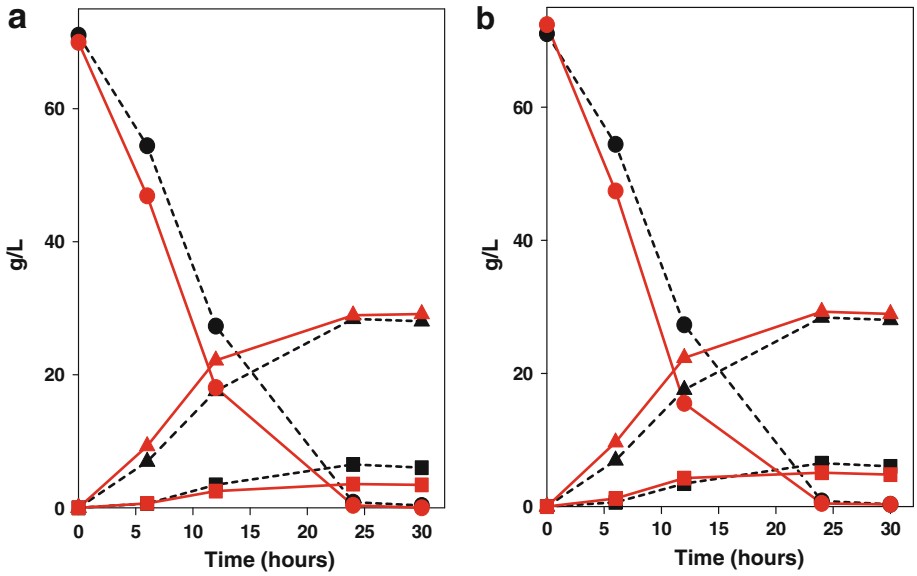

Fig. 4 Xylose consumption and products formation during fermentation by 424A(LNH-ST) strains. The (100 ml) YEPX medium contained xylose (70 g/l). **a** 424A(LNH-ST) (*dashed line*) and 424A(LNH-ST)/p*Kl*GAPDH (*solid line*), **b** 424A(LNH-ST) (*dashed line*) and 424A(LNH-ST)/p*Sc*4PPP (*solid line*). Xylose consumption (*filled circles*), xylitol production (*filled squares*) and ethanol production (*filled triangles*). The figure illustrates one of the representatives of duplicate experiments for each strain

Table 3 Product yields during micro-aerobic fermentation

Strains	Plasmids	Final cell density[a]	Xylose	Metabolic yield (in %)[b,c]		
		Dry weight (g/l)	Consumption (in %)	Xylitol	Glycerol	Ethanol
4124	p*Ps*XR-*Ps*XD-*Sc*XK	7.14	99.7 ± 0.4	7.3 ± 0.2	6.7 ± 0.3	76.8 ± 1.3
	p*Nc*XR-*Ps*XD-*Sc*XK	7.14	99.0 ± 0.4	9.0 ± 0.5	6.2 ± 0.2	78.6 ± 1.
	p*Cp*XR-*Ps*XD-*Sc*XK	6.24	15.2 ± 0.4	6.1 ± 0.2	0	34.2 ± 0.6
	p*Ps*XR-*Ps*XDm-*Sc*XK	5.95	70.8 ± 1.6	52.3 ± 3.5	1.9 ± 0.2	30.8 ± 0.6
	p*Nc*XR-*Ps*XDm-*Sc*XK	6.54	65.1 ± 9.0	22.4 ± 1.9	4.9 ± 0.8	60.1 ± 1.2
424A(LNH-ST)		7.38	99.1 ± 0.4	9.5 ± 0.2	5.4 ± 1.5	78.9 ± 0.6
	p*Kl*GAPDH	7.23	99.3 ± 1.0	5.5 ± 0.8	6.8 ± 0.3	82.2 ± 0.9
	p*Sc*4PPP	7.32	99.4 ± 0.1	7.2 ± 0.2	7.4 ± 0.6	79.6 ± 0.2

Each value represents the mean ± the standard error of two or three independent experiments

XR xylose reductase, *XD* xylitol dehydrogenase, *XDm* mutant xylitol dehydrogenase, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, *4PPP* four enzymes (*TAL1*, *TKL1*, *RKI1* and *RPE1*) from pentose phosphate pathway

[a] Cell density was measured by Klett meters and expressed in Klett units and then converted into dry weight. All fermentation experiments started with cell density 400 Klett units (corresponding to dry weight 4.76 g/l)

[b] During xylose fermentation, sometimes acetic acid was also produced. The amount of acetic acid was 5.6% of the consumed sugars in the case of 4124/p*Ps*XR-*Ps*XDm-*Sc*XK and no detectable acetic acid was observed in other cases

[c] Yields of fermentation products are expressed in the percentage of theoretical yield. For xylitol and glycerol, metabolic yield from xylose was calculated using the equation 100 × [amount of product, g]/[total amount of consumed sugar(s), g]. For ethanol, metabolic yield was calculated using the equation 100 × [amount of ethanol, g]/[total amount of consumed sugar(s), g × 0.51]

S. cerevisiae that can convert NADH to NADPH. It was reported that altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *S. cerevisiae* [30]. Correct combinations of XR and XD might/could solve the problem of imbalance of redox cofactors. Both XR and XD enzymes with different coenzyme specificity and catalytic efficiencies (k_{cat} etc.) were reported (Table 4). Some of them were from different sources [17, 31, 32] and others were (*Ps*XD) modified by site-directed mutagenesis [33]. The *Nc*XR from *N. crassa* is mainly NADPH dependent. The K_m values for NADPH and NADH for *Nc*XR were 9 and 21 μM, respectively, and *Nc*XR k_{cat} is two times higher compared to XR k_{cat} from *P. stipitis* (Table 4). *Cp*XR from *C. parapsilosis* is mainly NADH dependent but k_{cat} is similar to *Nc*XR (Table 4) [31]. Watanabe et al. [33] generated a *Ps*XD mutant by multiple site-directed mutageneses (D207A/I208R/F209S) that can also utilize $NADP^+$. Here, we have made three different XR-XD combinations having better coenzyme specificities; (1) NADPH-dependent XR (*Nc*XR) and $NADP^+$-dependent XD (*Ps*XDm) (2) NADH-dependent

Table 4 Coenzyme preference of XR and XD from various organisms or mutant enzymes

Organism	Enzyme	NADPH		NADH		NAD^+		$NADP^+$		References
		K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})	
P. stipitis	XR (*Ps*XR)	9	1,500	21	–	–	–	–	–	[17]
N. crassa	XR (*Nc*XR)	1.8	3,600	16	–	–	–	–	–	[32]
C. parapsilosis	XR (*Cp*XR)	36.5	–	3.3	3,100	–	–	–	–	[31]
P. stipitis	XD (*Ps*XD)	–	–	–	–	0.38	1,110	170	110	[33]
P. stipitis	XDm (*Ps*XDm)	–	–	–	–	1.30	320	0.90	2,550	[33]

XR xylose reductase, *XD* xylitol dehydrogenase, *XDm* mutant (D207A/I208R/F209S) xylitol dehydrogenase

XR (*Cp*-XR) and NAD^+-dependent XD (*Ps*XD) and (3) having dual cofactor dependent, NAD(P)H-dependent XR (*Ps*XR) and $NAD(P)^+$-dependent XD (*Ps*XDm). The xylose fermentation was compared with our original construct (having *Ps*XR-*Ps*XD-*Sc*XK). Single substitution was also tested with the replacement of *Ps*XR by the *Nc*XR gene. The *Sc*XK was present in all plasmids construct.

The replacement of *Ps*XR by *Nc*XR did show slight changes in xylose consumption and ethanol yield (Table 3). This indicates that the over-expressed *Nc*XR was slightly better than *Ps*XR, as evidenced by the aerobic growth on xylose (Fig. 1) and the more than 2% increase in ethanol metabolic yield (Table 3). However, production of xylitol in the strain carrying *Nc*XR was slightly increased (Table 3). On the other hand, the plasmid construct with the mutant xylitol dehydrogenase (*Ps*XDm) was much less active than the wild type *Ps*XD. The *Ps*XDm replacement increased the xylitol production by sevenfold and decreased the ethanol metabolic yield about 60% (Table 3). These results are in contradiction to results reported by Watanabe et al. [18] and Hou et al. [34]. Both authors reported significant decreases of xylitol production while using *Ps*XDm in their strains [18, 34]. The amount of xylitol production was decreased by 2.4-fold and the ethanol metabolic yield increased by twofold when *Ps*XR was replaced by the *Nc*XR enzyme. But overall *Nc*XR-*Ps*XDm was not a better combination of XR-XD than our original construct. The same results regarding xylose consumption and ethanol yield were reported by Hou et al. [34]. On the other hand, Watanabe et al. [18] reported that mutated *Ps*XDm had better ethanol yield as compared with the wild type *Ps*XD expressed in their strain and an additional mutation that increased thermostability by introducing a zinc binding site [35], yielding a significant improvement in xylose fermentation. There was no improvement of xylose fermentation using NADH-dependent XR (*Cp*XR). Both aerobic growth on xylose (Fig. 2) and xylose fermentation (Fig. 3) were very poor when the *Ps*XR was replaced by *Cp*XR.

Our next approach was to improve the xylose fermentation by increasing the NADPH pool. Vero et al. [6] showed that the over-expression of the *K. lactis* GDP1 gene, encoding an $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a xylose fermenting *S. cerevisiae* strain enhances ethanol production about 27%. Thus, NADPH formation appears to be necessary for better xylose fermentation. To reduce xylitol excretion during xylose fermentation, we used an approachsimilar to Jeppsson et al. [36]. With this approach, using our 424A(LNH-ST) strain, over-expression of *Kc*GAPDH enzyme decreased the xylitol production by more than 40% (Table 3).

Non-oxidative PPP is the only way to introduce xylulose 5-phosphate into the glycolysis. It was reported that the flux through the PPP is insufficient in *S. cerevisiae* compared to other yeast strains [21]. Over-expression of transaldolase form has been shown to improve growth on xylose in *S. cerevisiae* [21]. In contrast, over-expression of transketolase showed considerable reduction in growth on xylose [37]. Interestingly, over-expression of all four PPP genes in recombinant *S. cerevisiae* improves xylose consumption [38]. We tried to improve xylose fermentation using the same approach as described above (by over-production of non-oxidative four PPP enzymes transaldolase (TAL1), transketolase (TKL1), ribose-5-phosphate ketol-isomerase (RKI1) and ribulose-5-phosphate 3-epimerase (RPE1) (simply *Sc*4PPP)). Over-expression of four endogenous enzymes slightly improved on the consumption of xylose fermentation rate (Fig. 4), decreased the production of byproduct xylitol and slightly improved in the ethanol metabolic yield (Table 3).

Several research teams have successfully employed the strategies described above to improve xylose fermentation of their genetically modified strains [6, 10–13, 15, 16, 18, 19, 21, 34–38]. In our case, we did not see any significant improvement in xylose utilization or ethanol production using the same strategies as the others. One explanation for this phenomenon could be the different genetic background of the strains used. In our case we used the *S. cerevisiae* 4124

strain. Our original construct *S. cerevisiae* 424A(LNH-ST) [4, 10, 11] fermented xylose more efficiently compared to other genetically modified *S. cerevisiae* that use fungi pathways for xylose utilization and were subject to similar improvements as described in this article [6, 10–13, 15, 16, 18, 19, 21, 34–38]. It was suggested that besides the optimal cofactor usage, the "right combination or ratio of activities of enzymes" directly involved in xylose utilization (XR, XD, XK, PPP) could be needed to achieve efficient xylose fermentation [2, 12–16, 39]. In addition, Dowell et al. [40] recently suggested that same mutation in strains with different genetic backgrounds will lead to very different phenotypes. It is likely that this is true when the genetic changes are modifications of metabolic pathways through cloning. Based on our results, we suggest that strains with different genetic backgrounds could need quite different modifications to achieve efficient or improved xylose fermentation.

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