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BIOENERGY/BIOFUELS/BIOCHEMICALS

Improving ethanol and xylitol fermentation at elevated temperature through substitution of xylose reductase in *Kluyveromyces marxianus*

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Abstract Thermo-tolerant yeast *Kluyveromyces marxianus* is able to utilize a wide range of substrates, including xylose; however, the xylose fermentation ability is weak because of the redox imbalance under oxygen-limited conditions. Alleviating the intracellular redox imbalance through engineering the coenzyme specificity of NADPH-preferring xylose reductase (XR) and improving the expression of XR should promote xylose consumption and fermentation. In this study, the native xylose reductase gene (*Kmxyl1*) of the *K. marxianus* strain was substituted with XR or its mutant genes from *Pichia stipitis* (*Scheffersomyces stipitis*). The ability of the resultant recombinant strains to assimilate xylose to produce xylitol and ethanol at elevated temperature was greatly improved. The strain YZB014 expressing mutant *Ps*XR N272D, which has a higher activity with both NADPH and NADH as the coenzyme, achieved the best results, and produced 3.55 g l^{-1} ethanol and 11.32 g l^{-1} xylitol—an increase of 12.24- and 2.70-fold in product at 42 °C, respectively. A 3.94-fold increase of xylose consumption was observed compared with the *K. marxianus* YHJ010 harboring *KmXyl1*. However, the strain YZB015 expressing a mutant *Ps*XR K21A/N272D, with which co-enzyme preference was completely reversed from NADPH to NADH, failed to ferment due to the low expression. So in order to improve xylose consumption and fermentation in *K. marxianus*, both higher activity and co-enzyme specificity change are necessary.

Keywords Ethanol · *Kluyveromyces marxianus* · Corncob hydrolysate · Xylose reductase · Thermo-tolerant yeast

Introduction

D-Xylose is one of the main hydrolysis products of lignocellulosic biomass and the second most abundant fermentable material [42]. As the most abundant pentose in lignocellulose hydrolysates, xylose arguably holds the key to unlocking the production of value-added products from renewable biomass resources [5, 32]. In contrast to the efficient glucose fermentation in yeast, xylose fermentation has been challenging because only a few ethanol-producing microorganisms can readily ferment xylose, though many microorganisms utilize xylose as a carbon source [42].

Wild-type strains of *Saccharomyces cerevisiae*, the predominant organisms used for commercial ethanol production, are unable to utilize xylose and need to be genetically modified to produce ethanol from xylose [12, 35]. There are also native yeast species that ferment xylose to produce

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ethanol, including several *Pichia* and *Candida* species, as well as some strains of *Kluyveromyces marxianus* [38].

Because of its thermo-tolerance, high growth rate, and broad substrate spectrum, *K. marxianus* has attracted increasing attention [8]. *K. marxianus* can grow at elevated temperatures as high as 52 °C. It was reported that even at 45 °C, *K. marxianus* still can produce ethanol well with glucose [42]. One of the important potential applications of these strains is simultaneous saccharification and fermentation (SSF) of cellulosic biomass. The optimal temperature of cellulases used in SSF is 45–50 °C. While most yeast cannot survive at these temperatures, *K. marxianus* strains would allow SSF temperatures to be increased. The high fermentation temperature allows more rapid and efficient enzymatic cellulose hydrolysis [8, 42]. Most of the potential ethanologens used for industrial applications belong to the mesophilic group (28–35 °C), whereas thermophilic and thermotolerant ethanologens have certain advantages over mesophiles. Solvent tolerance, energy saving through reduced cooling cost, higher saccharification and fermentation rate, easier stripping of ethanol from broth, and minimum risk of contamination are the major advantages of thermophilic and thermotolerant ethanologens [20].

Many genetic engineering studies have been conducted to enable *S. cerevisiae* to ferment xylose and produce ethanol. Since 1993, with the introduction of *P. stipitis* xylose reductase (XR) and xylitol dehydrogenase (XDH) genes *Psxyl1* and *Psxyl2* the xylose fermentation of *S. cerevisiae* was improved [19, 34]. Other than the engineering of sugar transport [17], initial conversion of the pentose sugars [35], enhancing the pentose phosphate pathway (PPP) [14, 15], random improvements and evolutionary to improve xylose isomerase expression [21], modification of redox cofactors utilization, and improving the expression of XR and xylitol dehydrogenase (XDH) are also important [13, 15, 36].

Previous studies have shown that Lys21 is the only amino acid in *Ps*XR that form hydrogen bonds with NADPH but not with NADH in the binding pocket, and *Ps*XR's coenzyme specificity can be altered from NADPH and NADH to NADH alone by specifically targeted site-directed mutagenesis Lys21 to Ala21 [41]. The N272D mutant would also improve the catalytic efficiency [37, 41].

In the present study, we constructed several *K. marxianus* strains in which the XR from *K. marxianus* (*Km*XR) was substituted with XR from *Pichia stipitis* (*Ps*XR) or its mutants N272D [37] and K21A/N272D [41]. The resultant recombinant yeasts were characterized for their thermotolerance and ability to assimilate xylose and ferment xylose to ethanol and xylitol. Finally, the recombinant strain, which expressed *Ps*XR N272D mutant, was used to produce ethanol and xylitol with the corncob acid hydrolysate hemicellulose fraction.

Materials and methods

Reagents and microorganisms

All chemicals used were of analytical grade or higher. D-Xylose, yeast nitrogen base without amino acids (YNB) and dNTPs were obtained from Sangon Biotech Co. (Shanghai, China); D-glucose, D-galactose, D-arabinose, D-mannose, D-fructose, NADH, and NADPH were obtained from Bio Basic, Inc. (Toronto, Canada). Restriction enzymes and modifying enzymes were obtained from Fermentas Life Sciences (Fermentas China, Shenzhen, China). The yeast extract and peptone were purchased from Oxoid (Netherlands). Corncob was purchased from Hebei province, China. *K. marxianus* NBRC1777 and *P. stipitis* NBRC10063 were obtained from NBRC (Tokyo, Japan). *K. marxianus* YHJ010 was the *trp1*, *leu2*, *ura3* auxotrophic strain of NBRC1777 [11]. *K. marxianus*YZB001 was obtained through disrupting the *Kmxyl1* gene in YHJ010 [42]. Synthetic dropout (SD) medium (6.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose) supplemented with appropriate amino acids was used to select the transformants. Yeast extract/peptone-dextrose (YPD) medium (20 g/l peptone, 10 g/l yeast extract, and 20 g/l glucose) was used to aerobically culture *K. marxianus* strains. Fermentation and xylose assimilation ability was determined in YPX medium (20 g l^{-1} D-xylose, 10 g l^{-1} yeast extract, 20 g l^{-1} bacterial peptone). To prepare solid plates of each medium, 1.5 % agar was added. *Escherichia coli* XL10-Gold was used for cloning, and it was grown in Luria–Bertani (LB) medium.

Construction of the plasmids for expressing wild type *Psxyl1* or its mutant genes in *K. marxianus* YZB001

To substitute *Kmxyl1*in *K. marxianus*, the xylose reductase gene *Psxyl1* from *Pichia stipitis* was transformed into *K. marxianus* YZB001 [42], a *Kmxyl1* disrupted strain of *K. marxianus* YHJ010 [11]. *Psxyl1* gene was amplified from the genomic DNA of *P. stipitis* NBRC10063 with primers PSXR-KPN-F/PSXR-NOT-R (Table 1) and inserted into *Kpn*I and *Not*I sites of plasmid YEGAP [10]. The expression cassette including the GAPDH promoter, *Psxyl1,*and terminator was amplified with the primers GAP-P-F/GAP-T-R (Table 1) and inserted into the plasmid pKmUra3 [11] at the *Stu* I site to form the plasmid pPSXRPTU (Fig. s1) (Table 2). A plasmid named pPSXRPTUM1 containing the gene of PSXR mutant N272D was constructed from pPSXRPTU through site-directed mutagenesis of *Psxyl1* with the primers PSXRN272D-F/PSXRN272D-R (Table 1) as previously described [4]. Using the pPSXRPTUM1 as template, the double PSXR mutant PSXRK21A/N272D was constructed

Table 1 Primers used in the present study

Primer	Sequence[a]
PSXR-KPN-F	5′-CGGGGTACCATGCCTTCTATTAAGTTGAAC -3′
PSXR-NOT-R	5′-ATAAGAATGCGGCCGCTTAGACGAAGATAGGAATCTT -3′
GAP-P-F	5′-GAAGGCCTACCAGTTCTCACACGGAACA-3′
GAP-T-R	5′-GAAGGCCTTCAATCAATGAATCGAAAATGTCAT -3′
KMURA3-F	5′-ATTGGAAAGACCATTCTGCTTT-3′
KMURA3-R	5′-GGCAAAACGATGGATCGTTTT-3′
PSXRK21A-F	5′-GGTTTCGGCTGTTGGGCCGTCGACGTCGACACC-3′
PSXRK21A-R	5′- GGTGTCGACGTCGACGGCCCAACAGCCGAAACC-3′
PSXRN272D-F	5′-ATCATTCCAAAGTCCGACACTGTCCCAAGATTG-3′
PSXRN272D-R	5′-CAATCTTGGGACAGTGTCGGACTTTGGAATGAT-3′
RT-KMACT1-F	5′-CCCAATGAACCCAAAGAATAACAG-3′
RT-KMACT1-R	5′-GATAGCATGAGGCAAGGAGAAACC-3′
RT-PSXR-F	5′-GATGGTCTTCCCAAAGAGGCATTG-3′
RT-PSXR-R	5′-GTCGTTGAATCTCAAGTTGATGTC-3′

[a] Underlined, the restriction enzyme site

Table 2 Yeast strains and plasmids used in this study

Strain or plasmid	Relevant genotype	References
Strains		
K. marxianus yeast strains	Wild type *K. marxianus* form NBRC	[11]
NBRC1777		
YHJ010	NBRC1777,△*Kmura3::Kan*^{r}, Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG*	[11]
YZB001	YHJ010,△*Kmura3*::*Kan*^{r,} Δ*Kmleu2*::*hisG*, Δ*Kmtrp1*::*hisG*, Δ*Kmxyl1*::*Sctrp1*	[42]
YZB013	YZB001, *Kmura3*::pPSXRPTU	This study
YZB014	YZB001, *Kmura3*::pPSXRPTUM1	This study
YZB015	YZB001, *Kmura3*::pPSXRPTUM2	This study
Plasmids		
yEGAP	P_{Scgap}, T_{Scgap}, *Sctrp1*	[10]
pKmURA3(pHJ013)	*KmURA3*	[11]
pPSXRPTU	*Kmura3*, P_{Scgap}-*Psxyl1*-T_{Scgap}	This study
pPSXRPTUM1	*Kmura3*, P_{Scgap}-*Psxyl1*(N272D)-T_{Scgap}	This study
pPSXRPTUM2	*Kmura3*, P_{Scgap}-*Psxyl1*(K21A/N272D)-T_{Scgap}	This study

with the primers PSXRK21A-F/PSXRK21A-R (Table 1) and named pPSXRPTUM2.

Yeast transformation

The plasmids pPSXRPTU, pPSXRPTUM1, and pPSXRPTUM2 were linearized with *Sma*I digestion and transformed into *K. marxianus* YZB001 (Table 2) by the lithium acetate method [1, 40]. The YZB001 strain was cultured for 12 h at 37 °C in 5 ml of YPD medium, then collected by centrifugation at 5,000×*g* for 30 s, followed by suspension in 500 µl of one-step buffer (0.2 mM lithium acetate, 400 g l^{-1} polyethylene glycol 3,350, and 0.1 mM DTT). The yeast cells were collected by centrifugation again at 5,000×*g* for 5 s and resuspended in 100 µl of one-step buffer; 10 µl of *Sma*I digested plasmid was added and gently mixed. After incubation for 15 min at 47 °C, the suspension was mixed with 100 µl of distilled water and spread on the synthetic dropout (SD) medium. The transformants were selected on SD medium without uracil for 2 or 3 days at 37 °C, and the integration of *Ps*XRs were confirmed through PCR with primers PSXR-KPN-F/GAP-T-R (Table 1) [1, 40]. The obtained strains, which were transformed with *Psxyl1* or its mutant genes, were named YZB013, YZB014, and YZB015 (Table 2).

XR activity assay

The cells grown on the xylose fermentation medium at 37 °C were harvested by centrifugation at 10,000×*g* for 10 min. After the cells were washed with 100 mM potassium phosphate buffer (pH 7.0), they were resuspended in

the same buffer and then lysed by sonication (Vibra-Cell VC505,Connecticut, USA) for 20 min at 40 % power. The cell debris was separated by centrifugation at 10,000×*g* for 10 min, and the supernatant was then used to measure enzyme activity. The activity of XR was determined according to a previously described method in a spectrophotometer to monitor the change in A_{340} upon oxidation of NAD(P)H [42]. The assay mixture (1.0 ml) for the reaction contained 100 mM phosphate buffer (pH 7.0), 200 μM NADPH, 200 mM xylose, and enzyme solution (0.1 ml). The reaction was started by adding 0.1 ml of crude enzyme. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min under the specified conditions. The thermo-stability of the crude enzymes was also determined. YHJ010, YZB013, YZB014 and YZB015 strains were cultured at 37 °C for 36 h in YPX medium, then the cells were lysed and the supernatants were incubated in the mixture of ice and water, 42 or 45 °C water bath for 10 min, respectively. Then the retained activities were determined. All results were performed in triplicate and shown as mean values. The bars in the figures indicate the ranges of the standard deviation.

Fermentation with xylose and corn cob hydrolysate

For fermentation experiments, the recombinant yeast strains were first cultivated aerobically in YPX medium for 36 h at 37 °C. Cells were collected by centrifugation at 5,000×*g* for 5 min. The constructed strains were then inoculated into 30 ml of fermentation medium (YP medium with 20 g l^{-1} xylose, 30 g l^{-1} xylose, 40 g l^{-1} xylose or 25 g l^{-1} corncob hemicellulose hydrolysate xylose as the carbon source). The cell density in the fermentation medium was adjusted to an OD_{600} of 10. Anaerobic batch fermentation was carried out in the closed 50-ml bottles. The fermentations were performed at 250 rpm at 42 or 45 °C. All results were performed in triplicate and shown as mean values. The bars in the figures indicate the ranges of the standard deviation.

Analytical methods

The concentrations of D-xylose, xylitol, D-glucose and ethanol were analyzed by high-pressure liquid chromatography (HPLC) system (Agilent 1100, USA) with a ROA-Organic Acid H+ (8 %) column (Phenomenex, USA); 0.005 N H_2SO_4 was used as the mobile phase at a column temperature of 75 °C at a flow rate of 0.3 ml/min. The culture was centrifuged at 14,000×*g* at 4 °C for 5 min and the supernatant was analyzed by HPLC. Culture growth was monitored spectrophotometrically at 600 nm. At $OD_{600} = 1$, the concentration of the cells was equivalent to 0.411 g l^{-1} cells.

Preparation of corncob hemicellulose hydrolysate

The corncob hydrolysate was prepared by the method previously reported with some modifications [5, 6, 30]. Acid hydrolysis of corncobs was carried out at 110 °C with 1.0 % H_2SO_4 for 3 h using a solid:liquid (quantity:volume) ratio of 1:10. The residue was removed with filtration. The xylose in the hydrolysate was then concentrated by vacuum evaporation (1×10^4 Pa) below 70 °C, and the solution was neutralized with lime cream to a pH of 10.0. $CaSO_4$ produced during neutralization was removed immediately by filtration. After the pH value was adjusted to 6.0 with phosphoric acid, the hydrolysate was stored at 4 °C overnight, and the $Ca_3(PO_4)_2$ precipitate was removed by filtration. Charcoal absorption was used to decolorize and remove most of the toxin (acetic acid, furfural, and hydroxymethylfurfural (HMF)) in the hydrolysate. The decolorization conditions were performed at 80 °C with 200 rpm agitation for 15 min. The charcoal was removed by filtration, and the hydrolysate was stored at 4 °C [5, 6].

Real-time PCR analysis

The relative expression levels of *Ps*XRs were determined by real-time PCR (RT-PCR). Total RNA from cells, which had been cultivated in YPD or YPX media at 37 °C for 36 h, was isolated with yeast total RNA extraction kit (Sangon Biotech Co. Shanghai, China). Isolated RNA was treated with RNase-free DNase I (Toyobo, Japan) at 37 °C for 15 min to remove the potentially contained genomic DNA. cDNA was synthesized by ReverTra Ace qPCR RT Master Mix kit (Toyobo, Japan). Reverse transcription reaction was performed on an Arktik thermal cycler (Thermo Fisher, Massachusetts, USA) at 37 °C for 15 min, 50 °C for 5 min and denaturing at 98 °C for 5 min. The synthesized cDNA was quantitatively determined by Nanodrop 2000 (Thermo Fisher, Massachusetts, USA). Real-time PCR was conducted on a Bio-Rad iCycler iQ (Bio-Rad, USA) with THUNDERBIRD SYBR qPCR mix kit (Toyobo, Osaka, Japan). Gene *Kmact1* for actin was used as an internal control. The primers of *Kmact1* and *Psxyl1 s* for RT- PCR were shown in Table 1.

Amplification efficiency of real-time PCR was analyzed according to the protocols of Shi et al. [33]. Briefly, cDNA reversely transcribed from the total mRNA were used as the template for real-time PCR to generate a plot of log copy numbers of the tested mRNA versus the corresponding cycle threshold (C_T). The slope of the linear plot is defined as $-(1/\log E)$, where E is the amplification efficiency, and its value should approach 2 if the efficiency reaches the maximum [24]. Thus, the quantity of mRNA, relative to a reference gene, can be calculated using the formula $2^{-\Delta CT}$, where $\Delta C_T = (C_{T\ mRNA} — C_{T\ reference}\ RNA)$ [33]. Comparison of

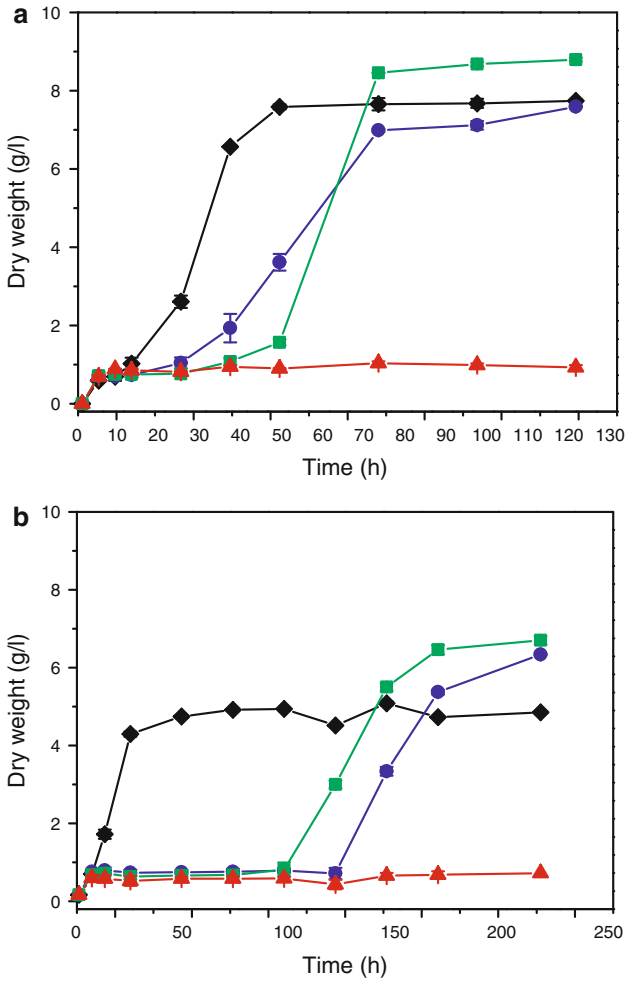


Fig. 1 The growth curve of recombinant strains at 42 °C (**a**) and 45 °C (**b**). YHJ010 (*filled diamond*), YZB013 (*filled circle*), YZB014 (*filled square*) and YZB015 (*filled triangle*)

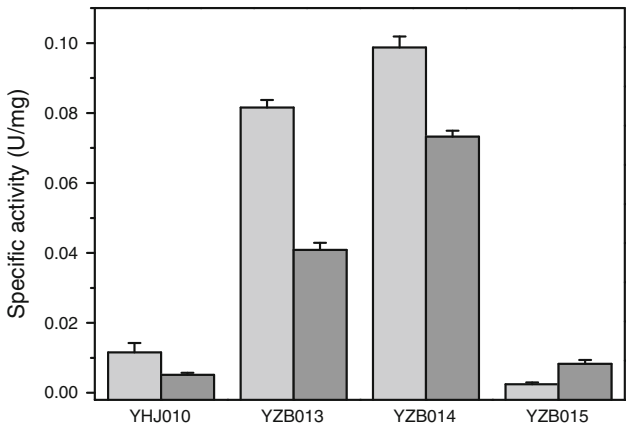


Fig. 2 The enzyme activity of the crude XR in recombinant strains. *Light-gray bars* and *dark-gray bars* indicate NADPH- and NADH-preferring XR activities, respectively

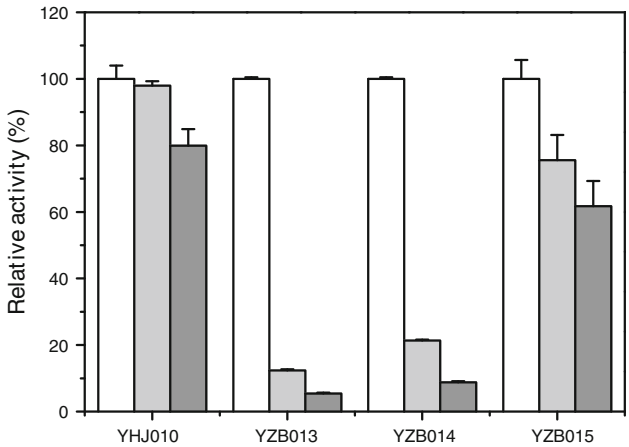


Fig. 3 The thermo-stability of the crude XR from various strains. *White*, *light-gray* and *dark-gray bars* indicate the retained activity after the incubation in the mixture of ice and water, at 42, and 45 °C respectively

mRNA expression was based on a comparative C_T method ($\Delta\Delta C_T$) [24, 39], and the relative mRNA expression can be quantified according to the formula of $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\ mRNA}—C_{T\ reference\ RNA})—(C_{T\ calibrator}—C_{T\ reference\ RNA})$ [24, 39]. The mRNA sample with the lowest C_T value, and thus the highest expression level, was selected as the calibrator, of which expression level represents 1 for normalization in each comparison. All results were performed in triplicate and shown as mean values. The bars in the figures indicate the ranges of the standard deviation.

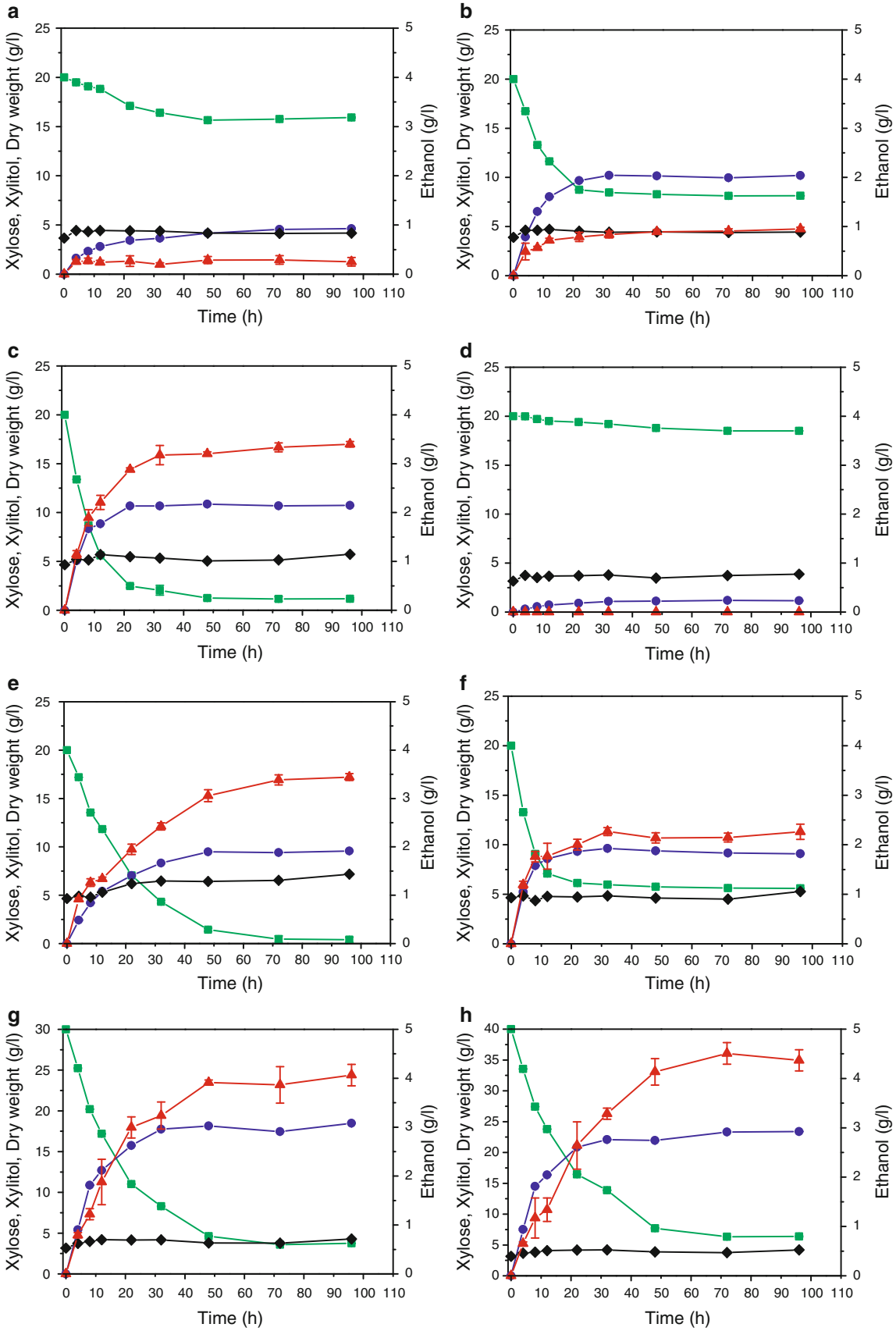
Results

Construction of *K. marxianus* strains expressing *Psxyl1* genes

The expression cassettes were successfully transformed into *K. marxianus* YZB001, and the transformants were selected on SD plates not containing uracil. The linearized plasmids including *Ps*XR expression cassettes were integrated into the genome of YZB001 and the transformants were confirmed by PCR with genomic DNA as template (Fig. s2). The strains obtained were named YZB013, YZB014 and YZB015 (Table 2), expressing *Ps*XR, *Ps*XR N272D and *Ps*XR K21A/N272D respectively.

Cultivation of the constructed strains at elevated temperatures

The *K. marxianus* YZB013, YZB014, and YZB015 strains were cultivated at 42 °C (Fig. 1a) and 45 °C (Fig. 1b) with YHJ010 (Table 2) as control. YZB013 and YZB014, grown in YPX medium, had a dry weight of over 7 g l^{-1} after 72 h of cultivation, but compared with YHJ010, a


a
b
c
d
e
f
g
h
Xylose, Xylitol, Dry weight (g/l)
Ethanol (g/l)
Time (h)

◀ **Fig. 4** The fermentation of xylose with the strains constructed in this study. Fermentation of YHJ010 (**a**), YZB013 (**b**), YZB014 (**c**), YZB015 (**d**) at 42 °C and YZB14 at 30 °C (**e**) or 45 °C (**f**) with YP medium and 2 % xylose; Fermentation of YZB014 at 42 °C with YP medium containing 3 % (**g**) and 4 % (**h**) xylose was determined. Xylose (*filled square*), xylitol (*filled circle*), ethanol (*filled triangle*) and dry weight (*filled diamond*)

growth delay was detected. At 42 °C, the recombinant strains YZB013 and YZB014 had a longer lag phase (about 24 h) and they reached stationary phase at 72 h, while YHJ010 had a shorter lag phase (about 12 h) and it reached stationary phase at 48 h. At 45 °C, the growth delay was more obvious and the YZB013 and YZB014 strains reached stationary phase at 216 and 168 h, respectively, which were 168 and 120 h later than YHJ010 which was almost not affected by the increased temperature (Fig. 1b). YZB015 could only grow to around 2 of the OD 600 with YPX at 42 and 45 °C (Fig. 1). To determine whether the growth delay was due to the weak activity of XR, YZB014 was cultured at 45 °C, and the XR activity at 96 h (lag phase), 120 h (exponential phase), and 144 h (stationary phase) was measured. The results (3.8, 60 and 32 mU mg^{-1}, respectively) indicated that the XR activity changes were consistent with the growth curve, so the delay is due to the lack of xylose reductase activity.

The activity of the crude XR in recombinant strains

The XR activity in the recombinant strains was determined with crude enzymes using NADH or NADPH as the coenzyme. The results showed that the XR activity of YZB013 improved 7.05- and 7.98-fold with NADPH (82 mU mg^{-1}) and NADH (41 mU mg^{-1}) as coenzymes, respectively, while XR activity in YZB014 improved 8.54- and 14.32-fold (99 and 73 mU mg^{-1}), respectively, compared to YHJ010 (12 and 5 mU mg^{-1}). However, the XR activity of YZB015 (2 and 8 mU mg^{-1}) improved only slightly (1.63 folds) when using NADH as the coenzyme (Fig. 2).

The thermo-stability of the crude XR in recombinant strains

The crude XR enzymes from the recombination strains were incubated at 42 or 45 °C for 10 min with NADPH as the coenzyme to determine the thermo-stability. After the incubation, the wild type XR of *K. marxianus* remained at 97.97 and 79.91 % activity, respectively, which was more thermal tolerant than the XRs from *P. stipitis* (YZB013 remained at 12.33 and 5.42 %; YZB014, 21.33 and 8.81 %; and YZB015, 75.53 and 61.70 %, respectively) (Fig. 3). The stability of *Ps*XR and *Ps*XR N272D was weaker than that of *Km*XR and *Ps*XR K21A/N272D.

Ethanol and xylitol production from xylose using *K. marxianus* strains containing *Ps*XR genes

To examine the xylose fermentation ability of the YHJ010, YZB013, YZB014, and YZB015 strains, fermentation was performed in YP medium containing 20 g l^{-1} xylose at 42 °C after aerobic cultivation of cells in YPX medium for 36 h at 37 °C. As shown in Fig. 4, YHJ010 consumed 4.82 g l^{-1} xylose and produced 4.20 g l^{-1} xylitol and 0.29 g l^{-1} ethanol in 48 h (Fig. 4a). YZB013 consumed 12.26 g l^{-1} xylose and produced 10.20 g l^{-1} xylitol and 1.10 g l^{-1} ethanol in 48 h (Fig. 4b). YZB014 consumed 19.00 g l^{-1} xylose and produced 11.32 g l^{-1} xylitol and 3.55 g l^{-1} ethanol in 32 h (Fig. 4c). YZB015 consumed xylose at a very low rate and did not produce ethanol (Fig. 4d). Therefore, the expression of *Ps*XR or *Ps*XR N272D, especially the expression of *Ps*XR N272D, can dramatically improve xylose consumption and xylitol and ethanol production.

There are several reports about fermentation with xylose at temperatures above 40 °C [3, 20, 31, 38]. Most of the strains are *K. marxianus,* which can ferment xylose up to 45 °C with the exception of the following two cases (Table 3). *Kluyveromyces* sp. IIPE453 consumed 17.65 g l^{-1} xylose and produced 1.75 g l^{-1} ethanol with an ethanol productivity of 0.025 g l^{-1} h^{-1} at 50 °C [20]. Another thermo-tolerant yeast *Hansenula polymorpha* was genetically engineered to improve xylose fermentation ability too [7]. When a mutated XR, a native XDH and XK were expressed in *H. polymorpha*, only 0.054 g l^{-1} h^{-1} of productivity is achieved and approximately 1.3 g l^{-1} ethanol is produced at 48 °C. The ethanol productivity of YZB014 in this study is higher than that of other strains, and it produces more ethanol than other strains at elevated temperatures (Tables 3, 4).

The influence of temperature and xylose concentration to the fermentation of *K. marxianus* with YZB014 strain

To evaluate the influence of temperature, fermentation was determined at various temperatures. Because of the best xylose fermentation ability at 42 °C, *K. marxianus* YZB014 was selected to conduct the fermentation at 30, 42, and 45 °C. The YZB014 consumed 19.60 g l^{-1} xylose and produced 9.57 g l^{-1} xylitol and 3.44 g l^{-1} ethanol at 30 °C (Fig. 4e), consumed 19.00 g l^{-1} xylose and produced 11.32 g l^{-1} xylitol and 3.55 g l^{-1} ethanol at 42 °C (Fig. 4c), and consumed 14.40 g l^{-1} xylose and produced 9.10 g l^{-1} xylitol and 2.26 g l^{-1} ethanol at 45 °C (Fig. 4f) (Table 4). These results indicated that up to 42 °C, YZB0104 had similar ethanol and xylitol productivity, and only decreased a little at 45 °C. That's because the xylose reductase activity increased as the temperature elevated though its stability decreased, and the ethanol and xylitol

productivity significantly increased at 42 °C compared to 30 °C (Table 4).

To analyze the influence of xylose concentration, fermentations with YP medium containing xylose at various concentrations were conducted with YZB014. During cultivation with 20 g l^{-1} xylose, YZB014 consumed 19.00 g l^{-1} xylose and produced 11.32 g l^{-1} xylitol and 3.55 g l^{-1} ethanol at 42 °C (Fig. 4c). However, YZB014 consumed 26.24 g l^{-1} xylose and produced 18.48 g l^{-1} xylitol and 4.07 g l^{-1} ethanol with 30 g l^{-1} xylose (Fig. 4g), after consuming 33.62 g l^{-1} xylose and producing 23.40 g l^{-1} xylitol and 4.51 g l^{-1} ethanol with 40 g l^{-1} xylose (Fig. 4h) at 42 °C. Therefore, as the xylose concentration increased, the amount of xylose consumed and xylitol and ethanol produced also increased; however, xylose consumption ratio decreased (Table 4).

Fermentation with the corncob hydrolysis production as carbon source

It is well known that corncob hemicellulose hydrolysate fermentation is complex because the hydrolysate contains several chemical compounds that are toxic (acetic acid, furfural, and HMF) to the yeasts [5]. After treatment with charcoal absorption, most of the toxic substances were removed, and mostly xylose remained. The concentrations of xylose, glucose, arabinose, and fructose were 25.88, 3.67, 3.08 and 1.33 g l^{-1}, respectively, in the total recovered hydrolysate. The constructed strain YZB014 fermented with this hemicellulose hydrolysate, consumed 21.41 g l^{-1} xylose, all glucose and fructose, and produced 12.21 g l^{-1} xylitol and 4.09 g l^{-1} ethanol at 42 °C (Table 4).

RT-PCR analysis

The low XR activity (Fig. 2) is the possible reason which led strain YZB015 failed to grow in the xylose medium. In order to verify whether the low activity was due to the low expression of the mutant *Ps*XR K21A/N272D, real-time PCR was performed. The expression profiles indicated that in YPD media, *Ps*XR expression level of YZB013 and YZB014 were similar (expression level around 0.03), but YZB015 expression level was only 0.007. Though when cultured with YPX media,

*Ps*XRs expression levels of YZB013 and YZB014 were improved greatly (0.46 and 1.00 respectively), *Ps*XR expression level of YZB015 was still at a low level (0.11) (Fig. 5).

Discussion

Most xylose-assimilating yeasts, including *K. marxianus,* have a xylose metabolic pathway in which xylose reductase converts D-xylose to xylitol. XR from *K. marxianus* NBRC1777 has sole coenzyme specificity, which is only activated with the NADPH cofactor [42]; however, the XDH in *K. marxianus* prefers to use NAD^+ [25]. To alleviate the imbalance of redox during xylose fermentation under oxygen-limited conditions, which is led by the coenzyme bias as mentioned above, the XR gene (*PsXyl1*) and its mutations from *P. stipitis* were used to replace *Kmxyl1* in *K. marxianus* to improve xylose consumption, and consequently increased ethanol and xylitol production.

The mutant K270R of *Ps*XR shows decreased NADPH-preferring activity without changing the NADH-preferring activity. Watanabe et al. [37] expressed the *Ps*XR K270R mutant together with a wild type *Ps*XDH gene in *S. cerevisiae*. The xylitol production was reduced by 31 %, and ethanol production was increased by 5.1 %, but only 2.26 g l^{-1} ethanol was produced from 5 g l^{-1} glucose and 15 g l^{-1} xylose [37]. Another mutant K270 M of *Ps*XR at the same position has a 17 times higher *Km* for NADPH but an unchanged *Km* for NADH with xylose as substrate when expressed in *S. cerevisiae*. These results show that K270 M could improve the ethanol yield and reduce the xylitol yield [13]. These results also showed that higher XR activity can improve the uptake of xylose. The mutant N272D of *Ps*XR has an increased ratio of NADH/NADPH utilization and V_{max} of a higher magnitude compared to the native enzyme. Thereby, the presence of the *Ps*XR N272D mutation increases xylose uptake and anaerobic growth [28]. The mutant K21A/N272D exhibits complete reversal of coenzyme specificity (from NADPH to NADH). Therefore, in our research, *Ps*XR N272D and K21A/N272D mutants were chosen to improve the xylose consumption and ethanol fermentation.

Growth and fermentation at elevated temperature have many advantages. The fermentation efficiency of YZB014 improves with increasing temperature (Table 4). The ethanol and xylitol productivity could reach 0.13 and 0.48 g l^{-1} h^{-1}, respectively, with a xylose consumption rate of 0.80 g l^{-1} h^{-1} and 87.62 % xylose consumption in 22 h at 42 °C (Fig. 5b), while the ethanol and xylitol productivity are only 0.089 and 0.32 g l^{-1} h^{-1}, respectively, with a xylose consumption rate of 0.59 g l^{-1} h^{-1} and 64.80 % xylose consumption in 22 h at 30 °C (Fig. 5e; Table 4). With an extended fermentation time, ethanol production is 3.40 and 3.44 g l^{-1} respectively. Therefore, fermentation at 42 °C accelerates the procedure and does not reduce the production. The YZB014 strain converts almost all the xylose to xylitol and ethanol at 30 and 42 °C, respectively (Table 3). This can be attributed to the high XR activity in the recombination strain. Although the fermentation ability of YZB014 is reduced at 45 °C compared to 42 °C, the ethanol productivity still increases to 0.15 g l^{-1} h^{-1} (Fig. 5f; Table 4). Therefore, if thermo-

Table 3 Comparison of xylose consumption and ethanol formation among various yeast strain

Strains	Temperature (°C)	Xylose ($g\ l^{-1}$)	Xylose consumption ($g\ l^{-1}$)	Xylitol production ($g\ l^{-1}$)	Xylitol yield ($g\ g^{-1}$)	Ethanol production ($g\ l^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Ethanol productivity ($g\ l^{-1}\ h^{-1}$)	Time of fermentation (h)	References
K. marxianus SUB-80-S	35	20	20	NR	NR	5.6	0.28	0.12	48	[26]
K. marxianus 80-SM-16-10	35	20	20	NR	NR	5.2	0.26	0.11	48	[26]
K. marxianus IMB4	45	10	4.62	NR	NR	1.2	0.26	NR	NR	[2]
K. marxianus IMB4	40	20	13.61	7.36	0.54	2.08	0.15	0.022	96	[38]
K. marxianus IMB4	45	20	8.68	2.57	0.30	0.65	0.07	0.0090	72	[38]
K. marxianus DMKU3-1042	30	20	20	~4.5	~0.23	2.6 ± 0.4	0.13 ± 0.02	0.036 ± 0.005	72	[31]
K. marxianus DMKU3-1042	40	20	20	~6.5	~0.33	2.2 ± 0.2	0.11 ± 0.01	0.046 ± 0.001	48	[31]
K. marxianus DMKU3-1042	45	20	~16	~3	~0.19	0.96 ± 0.16	0.06 ± 0.01	0.017 ± 0.001	72	[31]
Kluyveromyces sp. IIPE453	50	20	~17.5	11.5 ± 0.4	0.66 ± 0.02	1.75 ± 0.05	0.10 ± 0.01	0.025 ± 0.001	80	[20]
H. polymorpha CBS4732	48	120	~16	0.02 ± 0.0088	0.00125 ± 0.00055	1.31 ± 0.06	0.08 ± 0.004	0.054 ± 0.003	24	[7]
S. cerevbiae PUA6-9	30	20	19.65	9.88	0.50	3.08	0.16	0.04	76	[19]
S. cerevbiae TMB 3057	30	50	39.6 ± 3.4	8.71 ± 1.19	0.22 ± 0.03	13.30 ± 1.70	0.33 ± 0.02	0.133 ± 0.017	100	[16]
K. marxianus YZB014	30	20	19.60 ± 0.10	9.57 ± 0.30	0.49 ± 0.02	3.44 ± 0.06	0.18 ± 0.01	0.036 ± 0.001	96	This study
K. marxianus YZB014	42	20	19.00 ± 1.00	11.32 ± 0.36	0.60 ± 0.02	3.55 ± 0.19	0.19 ± 0.01	0.110 ± 0.006	32	This study
K. marxianus YZB014	45	20	14.40 ± 0.45	9.10 ± 0.21	0.63 ± 0.01	2.26 ± 0.10	0.16 ± 0.01	0.071 ± 0.003	32	This study

If the literature described several strains, only the best one is shown and the fermentation of mixed sugars (glucose and xylose) is not shown. The data without the standard deviation were due to no standard deviation provided in the original paper

NR not reported

Table 4 Comparison of fermentation in the strains constructed for this study at various conditions

Strain	Temperature (°C)	Xylose ($g\ l^{-1}$)	Xylose consumption ($g\ l^{-1}$)	Xylose consumption ratio (%)	Xylitol production ($g\ l^{-1}$)	Xylitol yield ($g\ g^{-1}$)	Ethanol production ($g\ l^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Ethanol Productivity ($g\ l^{-1}\ h^{-1}$)	Time of fermentation (h)
Strains										
YHJ010	42	20	4.82 ± 0.86	24.10 ± 4.30	4.20 ± 0.12	0.87 ± 0.03	0.29 ± 0.04	0.06 ± 0.01	0.006 ± 0.001	48
YZB013	42	20	12.26 ± 0.36	61.30 ± 1.80	10.20 ± 0.45	0.83 ± 0.04	1.10 ± 0.04	0.09 ± 0.01	0.023 ± 0.001	48
YZB014	42	20	19.00 ± 1.00	95.00 ± 0.50	11.32 ± 0.36	0.60 ± 0.02	3.55 ± 0.19	0.19 ± 0.01	0.111 ± 0.006	32
YZB015	42	20	1.21 ± 0.58	6.05 ± 0.29	1.11 ± 0.04	0.91 ± 0.03	–	–	–	48
Temperatures										
YZB014	30	20	19.60 ± 0.10	98.00 ± 0.50	9.57 ± 0.30	0.49 ± 0.02	3.44 ± 0.06	0.18 ± 0.01	0.036 ± 0.001	96
YZB014	42	20	19.00 ± 1.00	95.00 ± 0.50	11.32 ± 0.36	0.60 ± 0.02	3.55 ± 0.19	0.19 ± 0.01	0.111 ± 0.006	32
YZB014	45	20	14.40 ± 0.45	72.00 ± 2.25	9.10 ± 0.21	0.63 ± 0.01	2.26 ± 0.10	0.16 ± 0.01	0.071 ± 0.003	32
Xylose concentrations										
YZB014	42	25.88 ± 0.19[a]	21.41 ± 0.97	82.73 ± 3.75	12.21 ± 0.48	0.57 ± 0.02	4.09 ± 0.16	0.19 ± 0.01	0.085 ± 0.003	48
YZB014	42	20	19.00 ± 1.00	95.00 ± 0.50	11.32 ± 0.36	0.60 ± 0.02	3.55 ± 0.19	0.19 ± 0.01	0.111 ± 0.006	32
YZB014	42	30	26.24 ± 0.29	87.47 ± 0.97	18.48 ± 0.22	0.70 ± 0.01	4.07 ± 0.28	0.16 ± 0.02	0.085 ± 0.005	48
YZB014	42	40	33.62 ± 0.43	84.05 ± 0.11	23.40 ± 1.28	0.70 ± 0.04	4.51 ± 0.22	0.13 ± 0.01	0.063 ± 0.005	72

[a] Xylose was produced from corncob hydrolysis

stable enzymes could be expressed well, fermentation above 45 °C may be further improved.

YZB014 is one of the best ethanol fermentation strains of xylose at elevated temperature (Table 3), although the ability is still not as high as *S. cerevisiae* at 30 °C. These results are consistent with the previous hypothesis that higher enzyme activity improves xylose consumption because the XR activities of the YZB013 and YZB014 are significantly stronger than that of YHJ010 (Fig. 2). The xylose consumption and fermentation ability of *K. marxianus* YZB015 is very weak, in which the mutant K21A/N272D of *Ps*XR is expressed. Although the coenzyme preference of K21A/N272D was changed to NADH from NADPH, the XR activity in the *K. marxianus* YZB015 is too weak (even weaker than the XR of YH010) (Fig. 2) to support the strain growth and fermentation. The low XR activity was due to the lower expression and had been verified by real-time PCR results (Fig. 5). Despite the fact that the properties of K21A/N272D have been determined, the enzyme used was not expressed in yeast as it was in *E. coli*. [41]; therefore, the expression in yeast such as *K. marxianus* needs to be optimized.

The recombinant strains grew poorer as temperature was elevated. A similar phenomenon is also detected in GAPDH promoter controlled *Orpinomyces* XI expression in *K. marxianus* (data not shown). It is possible that the delay of XR expression was due to the transcription controlled by GAPDH promoter at 45 °C. The weak expression of lipase at higher temperature with the GAPDH promoter was also reported [27], but this hypothesis needs more investigations of the promoter. Although the *Ps*XRs are not relatively thermo-stable, ethanol fermentation is not hindered up to 42 °C (Table 4). However, the fermentation of YZB014 decreases at 45 °C (Table 4). These results suggest that in order to improve xylose consumption above 45 °C, more thermo-stable xylose reductase may be required.

While there are some reports about producing ethanol from xylose by engineering *S. cerevisiae* expressing xylose isomerase (XI) [16, 18, 23, 29], comparison of the xylose-fermenting ability by XR-XDH- and XI-carrying recombinant *S. cerevisiae* strains reveal that the XR-XDH xylose utilization pathway is much more efficient than the XI pathway [16, 29]. The XR-XDH pathway in *K. marxianus* deserved a more detailed study. The xylose fermentation ability of *K. marxianus* YZB014 in this study is not as high as that reported for recombinant *S. cerevisiae* such as TMB 3057, which could fermented 50 g/l xylose and produced 13.3 g/l ethanol and 8.71 g/l xylitol [16] (Table 3), but the xylose consumption improved dramatically, and ethanol production also improved greatly. In addition to the *Ps*XR mutant N272D expression described above, the introduction and over-expression of other endogenous genes,

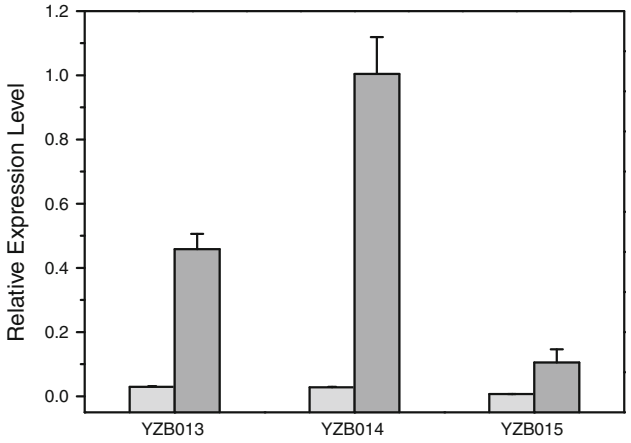


Fig. 5 Real-time PCR analysis of the expression of *Ps*XRs in recombinant strains YZB013, YZB014 and YZB015. *Light-gray bars* and *dark-gray bars* indicate glucose- and xylose-culture strains, respectively

including xylulokinase (x*yl3*), transketolase (*tkl1*), transaldolase (*tal1*) and sugar-transporter genes, which have been attempted to enhance the pentose phosphate pathway and/or xylose uptake [12], should be considered to achieve more effective ethanol production from xylose by recombinant *K. marxianus* at elevated temperatures.

Xylose released from lignocellulosic biomasses such as corncob through acid hydrolysis is a valuable substrate for xylitol and ethanol production [22]. Because of xylan, which accounts for up to one-third of the total carbohydrates, the fermentation of xylose favors the overall economics of the process of biomass utilization [9]. Therefore, the fermentation of the xylose in the corncob hydrolysate is very important. In this study, corncob hydrolysate was used to evaluate the fermentation ability of *K. marxianus* YZB014 (Table 4). The fermentation with YZB014 produced 4.09 g l^{-1} ethanol from the hydrolysate with 25.88 g l^{-1} xylose in addition to all the glucose and fructose at 42 °C, which is similar to that with pure xylose. Instead of pure xylose, other materials containing xylose such as hydrolysate from biomass would be used in the industrial application of xylose fermentation. In this study, the hydrolysate from corncob was used to evaluate the fermentation ability of YZB014.

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