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BIOENERGY/BIOFUELS/BIOCHEMICALS

Improved xylose fermentation of *Kluyveromyces marxianus* at elevated temperature through construction of a xylose isomerase pathway

Rongliang Wang · Lulu Li · Biao Zhang · Xiaolian Gao · Dongmei Wang · Jiong Hong

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Abstract To improve the xylose fermentation ability of *Kluyveromyces marxianus*, a xylose assimilation pathway through xylose isomerase was constructed. The genes encoding xylose reductase (*KmXyl1*) and xylitol dehydrogenase (*KmXyl2*) were disrupted in *K. marxianus* YHJ010 and the resultant strain was named YRL002. A codon-optimized xylose isomerase gene from *Orpinomyces* was transformed into *K. marxianus* YRL002 and expressed under GAPDH promoter. The transformant was adapted in the SD medium containing 1 % casamino acid with 2 % xylose as sole carbon source. After 32 times of trans-inoculation, a strain named YRL005, which can grow at a specific growth rate of 0.137/h with xylose as carbon source, was obtained. *K. marxianus* YRL005 could ferment 30.15 g/l of xylose and produce 11.52 g/l ethanol with a yield of 0.38 g/g, production rate of 0.069 g/l/h at 42 °C, and also could ferment 16.60 g/l xylose to produce 5.21 g/l ethanol with a yield of 0.31 g/g, and production rate of 0.054 g/l h at 45 °C. Co-fermentation with 2 % glucose could not improve the amount and yield of ethanol fermented from xylose obviously, but it could improve the production rate. Furthermore, *K. marxianus* YRL005 can ferment with the corn cob hydrolysate, which contained 20.04 g/l xylose to produce 8.25 g/l ethanol. It is a good platform to construct thermo-tolerant xylose fermentation yeast.

Keyword Thermo-tolerant · Elevated temperature · Xylose isomerase · Ethanol · *K. marxianus*

Introduction

Due to the depletion of fossil fuels and environmental problems such as global warming and acid rain, bio-ethanol from renewable lignocelluloses has attracted increasing attention. Conversion of all sugars to ethanol is therefore very important for the cost-effectiveness of the process [12]. Xylose is one of the major hydrolysis products of lignocellulosic biomass [23]. However, xylose cannot be efficiently fermented, in contrast to glucose [19]. Only a few ethanol-producing microorganisms can ferment xylose to some extent, even though many microorganisms utilize xylose as a carbon source.

In general, to utilize and ferment xylose, the xylose should first be converted into xylulose. Then, xylulose is phosphorylated into xylulose 5-phosphate by xylulokinase (XK), and the xylulose 5-phosphate is metabolized via the pentose phosphate pathway [45]. There are two different pathways for the conversion of xylose to xylulose. Xylose is converted to xylulose by xylose isomerase (XI) (E.C.5.3.1.5) in bacteria and some fungi or by xylose reductase (XR) (E.C.1.1.1.21) and xylitol dehydrogenase (XDH) in yeast (E.C.1.1.1.9). Redox imbalance resulting from the different co-enzyme preferences of xylose reductase and xylitol dehydrogenase under oxygen-limited

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R. Wang · L. Li · B. Zhang · X. Gao · D. Wang · J. Hong
School of Life Sciences, University of Science and Technology of China, Hefei, People's Republic of China

R. Wang · L. Li · B. Zhang · X. Gao · D. Wang (✉) · J. Hong (✉)
Hefei National Laboratory for Physical Science at the Microscale, Hefei 230026, Anhui, People's Republic of China
e-mail: dmwang09@ustc.edu.cn

J. Hong
e-mail: hjiong@ustc.edu.cn

conditions is one of the main reasons that xylose fermentation is suppressed in yeast [5]. The yeasts which can ferment xylose and produce ethanol were genetic modified to improve ethanol fermentation, such as in *Pichia stipitis* [6, 37–39] and *Candida utilis* [41]. However, these yeasts for ethanol production from xylose at the commercial level are limited, mainly due to the low ethanol tolerance, slow fermentation rates, carefully regulated oxygen requirement, and sensitivity to inhibitors [12]. *Saccharomyces cerevisiae*, which is more ethanol-tolerant and widely used in industry, was engineered to produce ethanol with xylose. There are several systemic reviews about this research [12, 13, 20].

Even after numerous attempts at engineering related enzymes and the reduction–oxidation pathway, fermentation of xylose remains elusive [45]. Comparing these two pathways (XI and XR-XDH), the isomerase pathway may produce more ethanol than the reduction–oxidation pathway, theoretically, because cofactor regeneration is not necessary. However, none of the attempts to express xylose isomerase gene (*XylA*) in yeast succeeded until Walfridsson expressed the *XylA* gene from *Thermus thermophilus* in *S. cerevisiae* [46]; however, the resulting expression was not strong enough to support ethanol fermentation with xylose. A fungal XylA gene from *Piromyces* sp. E2 (ATCC 76762) that was isolated from the feces of an Indian elephant was functionally expressed in *S. cerevisiae* [46], and after adaptation, the recombinant *S. cerevisiae* could grow at specific rates of 0.18 and 0.03/h under aerobic and anaerobic conditions, respectively. This research created an opportunity for the construction of isomerase pathways in yeast. Another fungal *XylA* gene from anaerobic fungus *Orpinomyces* sp., which was isolated from cattle rumen fluid, was cloned and expressed in *S. cerevisiae*. The recombinant strain could ferment xylose and produce ethanol without adaptation [5]. Brat et al. [5] functionally expressed a prokaryotic *XylA* gene from *Clostridium phytofermentans* in *S. cerevisiae* after screening 12 codon-optimized xylose isomerase genes. As shown in these successful cases, the application of XI has developed quickly recently.

Simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and cofermentation (SSCF) can increase yield and saccharification rate, reduce investment costs, and lower the contamination by the presence of ethanol [10]. Fermentation at an elevated temperature can accelerate the enzymatic hydrolysis during SSF and SSCF as well as reduce cooling and the ethanol distillation costs and also reduce the risk of contamination [28]. Sibirny's group expressed *Escherichia coli XylA* in thermo-tolerant yeast *Hansenula polymorpha* and evaluated its xylose fermentation ability; only 0.6 g/l ethanol at 48 °C and 1.03 g/l at 37 °C was produced [9]. They also tried to overexpress engineered xylose reductase, xylitol dehydrogenase, and xylulokinase in *H. polymorpha*, but the amount of produced ethanol was also small (about 1.6 g/l at 48 °C) [9]. *Kluyveromyces marxianus* is also a thermo-tolerant yeast that can survive at temperatures as high as 52 °C. It can grow and produce ethanol well from glucose, even at 45 °C [28]. In this study, growth and fermentation with xylose was carried out at 42 or 45 °C. The experiments at 42 °C were conducted to evaluate if YRL005 was more thermo-tolerant in xylose fermentation to other yeasts. Because *K. marxianus* 1777 could ferment with glucose rather well (0.49 g/g) at 45 °C, the experiments at 45 °C were used to determine if the strain RYL005 can also ferment xylose well. Also a *K. marxianus* strain exhibited resistance to aromatic compounds and HMF and this strain showed higher aldehyde assimilation rates in comparison to other fermentation microorganisms. It was shown that at compound concentrations found in the hydrolysate of pretreated poplar biomass (furfural 490 mg/l, catechol 30 mg/l, vanillin 18 mg/l, and 4-hydroxybenzaldehyde less than 10 mg/l), there was no toxic effect on growth and fermentation ability of this *K. marxianus* strain in experiments where inhibitors were added as single compounds [29]. However, the xylose-fermenting ability of *K. marxianus* is not high due to the redox imbalance generated under oxygen-limited conditions [15, 24, 48]. XR from *K. marxianus* NBRC1777 has sole coenzyme specificity, which is only activated with the NADPH cofactor [50]; however, the XDH in *K. marxianus* NBRC1777 prefers to use NAD^+ [24]. Substitution KmXR with XR and its N272D mutant from *Scheffersomyces stipitis* can improve xylose assimilation and fermentation in *K. marxianus*, but the xylose fermentation ability was still low [49]. To construct efficient yeast for SSF and SSCF, it is important to improve the xylose fermentation ability.

In this study, we deleted and re-established the xylose assimilation ability of *K. marxianus* through disruption of the xylose reductase and xylitol dehydrogenase genes and expression of a XI to avoid any redox imbalance. The resultant strain can assimilate xylose as its sole carbon source. It grows very well with a growth rate of 0.137/h at 42 °C, even as high as at 45 °C, a growth rate of 0.07/h was reached after a long lag phase (Fig. S1) under aerobic conditions, and can produce ethanol without detectable xylitol under anaerobic conditions. Corn cob, a type of agricultural waste, consists of approximately 45 % cellulose and 35 % hemicelluloses [34]. The main component of the acid hydrolysate of hemicelluloses from corn cob is xylose, which can be used to produce ethanol and xylitol [22]. In this study, the hydrolysate from corn cob was used as a model substrate to evaluate the constructed yeast.

Materials and methods

Chemicals and strains

All chemicals were reagent grade or higher and were purchased from Sangon Biotech Co. (Shanghai, China) unless otherwise noted. Yeast extract, peptone, and tryptone were purchased from Oxoid (Cambridge, UK). All restriction enzymes and T4 DNA Ligase were obtained from Fermentas Life Sciences (Fermentas China, Shenzhen, China). PrimeStar HS DNA polymerase was from TAKARA (Dalian, China). *E. coli* XL10 gold was used for all DNA manipulations. Luria–Bertani (LB) medium with 100 μg/ml ampicillin was used to cultivate *E. coli*. *K. marxianus* YHJ 010 [*Kmura3::Kan*^{r} *Kmleu2::hisG, Kmtrp1::hisG* auxotrophic strain derived from *K. marxianus* NBRC 1777 (NBRC, Japan)] was grown on solid or in liquid YPD media (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose). Synthetic dropout medium (SD) (6.7 g/l yeast nitrogen base, 2 % glucose) with supplied amino acids [36] was used to screen the yeast transformants. SD medium with 1 % casamino acids was used for the adaptation and to determine the xylose assimilation ability. *K. marxianus* YZB 001 [50] in which the xylose reductase gene (*KmXyl1*) was disrupted was used to disrupt the xylitol dehydrogenase gene (*KmXyl2*) and to construct the double gene disruption strain. The xylose ethanol fermentation was conducted in liquid YPX medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l, or 50 g/l xylose).

Construction of the xylose reductase and xylitol dehydrogenase gene double-disruption strain

To construct the double-disruption strain, the *KmXyl2* gene in *K. marxianus* YZB 001 was further disrupted. The *KmUra3* gene [14] amplified from plasmid pKmURA3 using primers URA3-MfeI-F and URA3-KpnI-R (Table 1) was inserted into the *KmXyl2* gene between the *Kpn* I and *Eco*R I sites. Afterwards, a fragment containing the disrupted *KmXyl2* and functional *KmUra3* genes was amplified by primers XDH-F and XDH-R (Table 1) and used as a disruption cassette for transformation into YZB 001, as previously described [14]. Double-disruption strains (YLUA 006) were screened on SD medium containing leucine.

To recover the URA3 selection marker, the *KmUra3* was disrupted again. A *KmUra3* DNA fragment, which was

Table 1 Primers used in this study

Primer	Sequence (5′ → 3′)[a]
XI-EcoRI-F	ACGTGAATTCGAAACGATGACCAAGGAAT ACTTCCCAACCATCGG
XI-NotI-R	ATAAGAATGCGGCCGCTTAGTGGTGGTGGT GGTGGTGTTGGTACATAGCAACGATAGC
XDH-F	ACCAACACTCAAAAAGCCG
XDH-R	TGGACCATCAATGATAGTCT
URA3-MfeI-F	AAACCGTTCAATTGGAATTCTGATTGGAAAGACCA
URA3-KpnI-R	GGGGTACCTCGGTGCAAAAAACAGCTTC
GAP-StuI-F	GAACCGGTACCAGTTCTCACACGGAAC
GAP-StuI-R	GAAGGCCTTCAATCAATGAATCGAAAATGTCAT
RT-KM-ACT-F	CCCAATGAACCCAAAGAATAACAG
RT-KM-ACT-R	GATAGCATGAGGCAAGGAGAAACC
RT-KM-GAPDH-F	GTCCAGAAAGAACATCGAAGTTGTC
RT-KM-GAPDH-R	GTAGCTGGGTCTCTTTCTTGGAAG
RT-XI-F	TCGAAGGTAAGGACTCTAAGAACC
RT-XI-R	CTTTTGCTTAGCGATAGCGATTGGG
RT-KM-XK-F	GAAACGAGTCCAGTCGATGTCTGG
RT-KM-XK-R	CTGTTTCTTTGTGCTGTGGTCTTGC
RT-KM-TKL-F	CTAAGTACTCTCACCAACAATTC
RT-KM-TKL-R	GAGAATAGACTTGCTTACCCTTG
RT-KM-TAL-F	GTTGTTGCCGACACTGGTGATTTCG
RT-KM-TAL-R	CTTGTCGACGGAAATCTCAACTTGC
RT-KM-RPE-F	CAGAAGTTCATGAGCGATATGATG
RT-KM-RPE-R	CCGCCGGGTCGTCCGATCGGAACAC
RT-KM-RKI-F	GCTGCATACCGTGCGGTTGATGAG
RT-KM-RKI-R	CTCAGGATATTGCTCAATGCTCCC

[a] Restriction enzyme sites are *underlined*

digested with Bst1107I to remove a 594-bp fragment from the ORF, was used as the disruption cassette to disrupt the *KmUra3* that was introduced into the genome during the previous step. The *KmUra3* gene re-disrupted strain (YRL 002) was selected on SD medium containing 0.1 % 5′-fluoro-orotic acid (5′-FOA).

Expression of the xylose isomerase gene in the double-disrupted strain

The codons of the *XylA* from *Orpinomyces* sp. were optimized based on the codon usage of *K. marxianus* and inserted into plasmid YEGAp at the *Eco*RI and *Not* I sites. Subsequently, an expression cassette that included the GAPDH promoter, *XylA*, and the GAPDH terminator was amplified by PCR using primers GAP-StuI-F and GAP-StuI-R (Table 1) and inserted into plasmid pKMURA3 at the *Stu*I site. The resultant plasmid was digested by *Spe* I and transformed into the double-disruption strain (YRL 002) as described previously [1]. Transformants (YRL 003) were selected on SD medium containing leucine.

Adaptation to improve xylose assimilation

After the strain transformed with the *XylA* was obtained, it was inoculated into a 15-ml tube containing 5 ml of SD medium and 10 g/l of casamino acids with 20 g/l xylose as the sole carbon source and then cultivated at 37 °C for 48 h to determine whether it could assimilate xylose. Afterwards, the strain was trans-inoculated into fresh adaptation medium (same as above) with a starting OD_{600} of 0.2. This step was repeated until the strain could grow well in the adaptation medium. The growth rate was then measured in a shake flask (a 250-ml flask containing 50 ml YPX medium or SDX medium) at 42 or 45 °C.

Fermentation of xylose

Xylose fermentation was conducted at 42 or 45 °C. *K. marxianus* YRL 005, YRL 003, YRL 002, or YHJ 010 (Table 2) was pre-cultured in 50 ml of YPD at 42 °C for 24 h and then the cells were recovered by centrifugation at room temperature at 5,000 $\times g$ for 3 min. After the cells were washed with sterilized water, the cells were inoculated into 40 ml of YPX (20 g/l or 50 g/l xylose) medium at a starting OD_{600} of 10 in a 50-ml anaerobic bottle supplied with butyl rubber stopper (Huakai Glassware Co., Jiangsu, China) to start the fermentation. The fermentations were conducted at 250 rpm and 42 or 45 °C for 192 or 120 h, and samples were taken every 24 h.

Corn cob hydrolysate preparation

Corn cobs were obtained from Tangshan of Hebei province in China and ground to the 30-mesh size to serve as the raw material. The corn cob hydrolysate was prepared as reported previously, with modifications [7]. Acid hydrolysis of corn cobs was conducted at 110 °C with 1 % (v/v) H_2SO_4 using a ratio of 1:10 [solid: liquid (w:v)] for 3 h. After the reaction was completed, the remaining solids were separated from the aqueous solution by filtration. The pH of the filtered hydrolysate was first adjusted to 10 with calcium oxide and then neutralized to pH 6 with phosphoric acid. After the hydrolysate was kept at 4 °C overnight, it was filtered to remove the $Ca_3(PO_4)_2$ precipitate. Charcoal absorption was used to decolorize and remove most of the fermentation inhibitors in the hydrolysate. The hydrolysate was mixed with activated charcoal (at a ratio of solid/liquid = 2.4 %) and agitated at 250 rpm, 42 °C for 1 h, after which the hydrolysate was recovered through filtration and used in the fermentation medium.

Table 2 Yeast strains used in this study

Strain	Relevant genotype	Reference
K. marxianus		
YHJ 010	*ΔKmura3::Kan*^{r}*ΔKmleu2::hisG, ΔKmtrp1::hisG*	[14]
YZB 001	*ΔKmura3::Kan*^{r} *ΔKmleu2::hisG, ΔKmtrp1::hisG Δxyl1::trp1*	[50]
YLUA 006	*ΔKmura3::Kan*^{r} *ΔKmleu2::hisG, ΔKmtrp1::hisG Δxyl1::trp1 Δxyl2::Kmura3*	This study
YRL 002	*ΔKmura3::Kan*^{r} *ΔKmleu2::hisG, ΔKmtrp1::hisG Δxyl1::trp1 Δxyl2:: ΔKmura3*	This study
YRL 003[a]	*ΔKmura3::Kan*^{r} *ΔKmleu2::hisG, ΔKmtrp1::hisG Δxyl1::trp1 Δxyl2:: ΔKmura3 KmUra3::xylA*	This study
YRL 005[a]	*ΔKmura3::Kan*^{r} *ΔKmleu2::hisG, ΔKmtrp1::hisG Δxyl1::trp1 Δxyl2:: ΔKmura3 KmUra3::xylA*	This study

[a] YRL005 was derived from YRL003 after adaptation

Fermentation of xylose in corncob hydrolysate

As described in the part "Fermentation of xylose", the cells of *K. marxianus* YRL 005 were recovered from pre-culture and inoculated into 40 ml of YP medium containing corn cob hydrolysate as the carbon source with a starting OD_{600} of 10 in an anaerobic bottle to initiate the fermentation. The fermentations were conducted at 250 rpm at 42 or 45 °C for 192 h, and samples were taken every 24 h.

Xylose, xylitol, xylulose, glucose, glycerol, acetic acid, furfural, and ethanol analysis

After the samples were taken and centrifuged, the concentrations of xylose, xylulose, glucose, glycerol, acetic acid, furfural, and ethanol in the supernatant were detected by HPLC using a Rezex ROA-Organic Acid H+ (8 %) column (Phenomenex, Torrance, CA, USA) with an Agilent 1200 refractive index detector (Agilent Technologies, Inc. Santa Clara, CA, USA). The system was operated at 75 °C with a flow rate of 0.3 ml/min and using 2.5 mM H_2SO_4 as the mobile phase.

D-xylose isomerase, D-xylose reductase, and D-xylitol dehydrogenase assay and protein analysis

To confirm that after genes disruption the XR-XDH pathway was deleted, the activity of XR and XDH had been determined in *Kmxyl1*-disrupted strain, and the XI activity was measured to confirm that *XylA* was functionally expressed. XI activity was measured as described by Madhavan [25], with some modifications. The yeast cells were harvested by centrifugation for 10 min at 15,000 $\times g$ at 4 °C. The cells were washed twice with 0.5 ml of sterilized water and then re-suspended in 1 ml of buffer A (50 mM Tris–HCl, 25 mM NaCl, pH 8.0). The cells were then lysed by sonication and centrifuged at 4 °C (20 min at 15,000 $\times g$), and the supernatant was used as the cell extract for the activity assay. D-xylose isomerase activity was assayed in a 275-µl solution containing 100 µl of enzyme solution, 50 mM of sodium phosphate buffer (pH 8.0) and 40 mM $MgCl_2$. The reaction was initiated by adding 20 mM xylose at 37 °C for 15 min and terminated by adding 25 µl 50 % (m/v) trichloroacetic acid (TCA). Xylulose production was quantified by the ketose determination method [8]. One unit of D-xylose isomerase was defined as the amount of enzyme that produced 1 µmol D-xylulose per min.

The activity of xylose reductase and xylitol dehydrogenase was determined according to a previous report [24]. The D-xylose reductase activity was assayed in a 1-ml solution containing 100 mM phosphate buffer (pH 7.0), 200 µM NAPDH, and enzyme solution (0.1 ml) at 25 °C. The reaction was initiated by adding 200 mM xylose. 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 xylitol dehydrogenase was mixed with 100 mM CAPSO buffer (pH 9.5), 0.5 mM NAD^+, and 400 mM xylitol at 25 °C. The reaction was initiated upon the addition of xylitol, and the absorbance at 340 nm was monitored with a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 µmol of NADH per min under the assay conditions. The protein concentration of the crude cell extract was measured using the method described by Bradford [4].

Real-time PCR analysis

The relative expression levels of xylose isomerase (*xylA*), xylulose kinase (*Kmxyl3*), transaldolase (*Kmtal1*), transketolase (*Kmtkl1*), D-ribulose-5-phosphate 3-epimerase (*Kmrpe1*), and ribose-5-phosphate ketol-isomerase (*Kmrki1*) were determined by real-time PCR (RT-PCR). Total RNA from these cells were isolated by 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 the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Japan). Reverse transcription reaction was performed on a Arktik thermal cycler (Thermo Fisher Scientific, Waltham, MA, 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 Scientific). Real-time PCR was conducted on a Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA, USA) with THUNDERBIRD SYBR qPCR mix kit (Toyobo, Osaka, Japan). Gene *Kmact1*, *gapdh* were used as an internal control. The primers for RT-PCR are shown in Table 1. Amplification efficiency of real-time PCR was analyzed according to the protocols of Shi et al. [40].

Results and discussion

Though many *K. marxianus* strains have been reported as capable of growth on xylose, their ability to ferment xylose to produce ethanol is weak with ethanol yield less than 0.28 g/g (theoretical yield 0.51 g/g) (Table 3). Additionally, the genetic modification of *K. marxianus* has been hindered by the lack of convenient genetic engineering tools. These are possible reasons that little research exists on xylose fermentation with *K. marxianus*, even though this yeast can ferment glucose well at temperatures as high as 45 °C with 0.49 g/g ethanol yield [14]. After a

Table 3 Comparison of xylose consumption and ethanol formation among various strains of *K. marxianus*

Xylose (g/l)	Xylose consumption (g/l)	Temperature (°C)	Ethanol yield (g/g)	Ethanol (g/l)	Ethanol productivity (g/l/h)	Time (h)	Strains	Reference
20	20	35	0.28	5.6	0.117	48	SUB-80-S	[26]
10	10	45	0.08–0.12	0.8–1.2	0.036–0.055	22	IMB3	[2]
20	13.61	40	0.15	2.08	0.022	96	IMB4	[48]
20	8.68	45	0.07	0.65	0.009	72	IMB4	[48]
20	20	NA	0.251	5.02	0.056	90	DMKU3-1042	[32]
20	20	30	0.13	2.6	0.036	72	NA	[33]
20	20	40	0.11	2.2	0.046	48	NA	[33]
20	~16	50	0.06	1.2	0.017	72	NA	[33]
20	17.24 ± 0.21	42	0.39 ± 0.021	6.74 ± 0.05	0.047	144	YRL005	This study
50	30.15 ± 0.38	42	0.38 ± 0.003	11.52 ± 0.19	0.069	168	YRL005	This study
20	7.25 ± 0.41	45	0.31 ± 0.009	2.27 ± 0.29	0.032	72	YRL005	This study
50	16.6 ± 0.11	45	0.31 ± 0.014	5.21 ± 0.07	0.054	96	YRL005	This study

If a reference contained several strains, only the best was 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

Values are presented as mean and standard deviation of three independent experiments

NA not available from the literature

triple-auxotrophic strain was obtained and the genes for xylose reductase and xylitol dehydrogenase were cloned, genetic modification of *K. marxianus* to improve xylose fermentation became possible [14, 24, 50].

Double xylose assimilation gene disruption

Most of the *K. marxianus* strains can grow well on xylose under aerobic conditions through a native xylose assimilation pathway, unlike *S. cerevisiae*, which lacks a complete xylose metabolic pathway; thus, it is possible to improve the xylose fermentation more conveniently in this species. Here, the *KmXyl1* and *KmXyl2* genes, which encode enzymes that have different co-enzyme preferences leading to redox imbalance under oxygen-limited conditions, were disrupted. After the *Kmxyl2* disruption cassette was transformed into *K. marxianus* YZB 001 and the transformants were selected on synthetic dropout (SD) medium, a double-gene-disrupted strain was obtained. The strain obtained was confirmed by PCR with genomic DNA as the template and designated YLUA 006. Subsequently, YLUA 006 was transformed with a *KmUra3* disruption cassette and screened on SD medium containing 5′-FOA. The *KmUra3*-disrupted strain obtained was designated YRL 002, and *KmXyl1*, *KmXyl2*, *KmUra3*, and *KmLeu2* were disrupted in this strain (Table 2). As to the activity of XR and XDH, the cells of each strain were cultivated in YPD medium at 42 °C for 12 h in which was exponential phase as pre-culture, then trans-inoculated into YPX culture and cultivated another 12 h. There was no activity of XR and XDH in YRL 002, YRL 003, and YRL 005, while 21.4 mU/mg (XR) and 0.72 U/mg (XDH) were detected in control strain YHJ 010. Also, during the fermentation, no xylitol accumulation was detected in YRL 002, YRL 003, or YRL 005, proved that there were no XR and XDH in these strains. Therefore, the XR-XDH pathway was completely disrupted.

Expression of xylose isomerase

The codon-optimized *XylA* from *Orpinomyces* sp. was transformed into *K. marxianus* YRL 002 and expressed under the GAPDH promoter. The strain obtained was named YRL 003 (Table 2). Aerobically cultivated in either SD or YPX medium, at 37 or 42 °C, respectively, the xylose assimilation ability of YRL 003 was better than YRL 002, but its growth was weak with xylose as carbon source (Fig. 1a, b). YRL 003 was then inoculated into SD casamino acid medium containing 2 % xylose as the sole carbon source and adapted to improve its xylose assimilation ability.

Adaptation to improve xylose assimilation

After 32 generations of adaptation at 37 °C, a strain named YRL 005 of which growth was suddenly increased with xylose as carbon source was obtained (Table 2). It was found to grow very well with xylose (Fig. 1a, b). In SD medium, it grew to OD_{600} 1.4 (Fig. 1a) at 37 °C with a growth rate of 0.108/h. In YPX medium, *K. marxianus* YRL 005 also grew well at 42 °C and reached an OD_{600} of 16 in 30 h under aerobic culture with a growth rate of 0.137/h in the exponential phase, while the growth of the

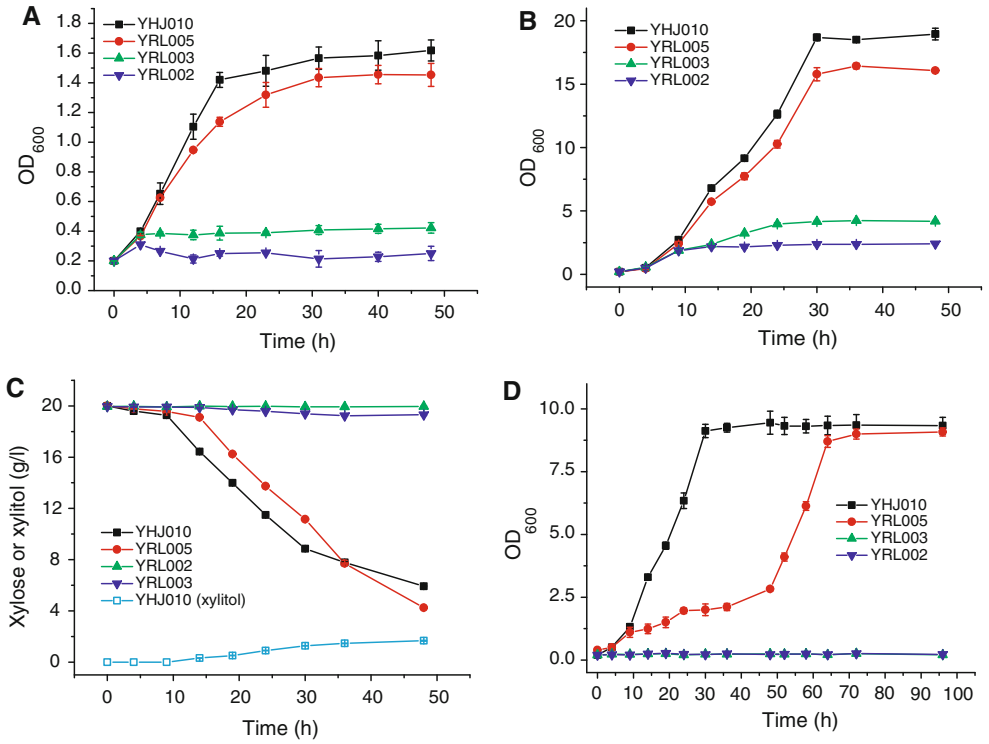

Fig. 1 Growth of *K. marxianus* strains with 2 % xylose as the carbon source under aerobic conditions. **a** Growth in liquid SD medium at 37 °C, **b** growth in YPX medium at 42 °C, **c** xylose consumption and xylitol formation at 42 °C with YPX medium, only YHJ010 produced xylitol, **d** growth in YPX medium at 45 °C. YRL 002: *KmXyl1* and *KmXyl2* double-disrupted strain; YRL 003: YRL 002 transformed with the *XylA* gene; YRL 005: strain resulting from adaptation of YRL 003. YHJ 010: *KmTrp1, KmLeu2, KmUra3* auxotrophic strain derived from NBRC 1777 (a detailed genotype of each strain is shown in Table 2)

double-disruption strain (YRL 002) and the strain before adaptation (YRL 003) were weak (Fig. 1a, b). In comparing the growth of YRL 005 in YPX and YPD at 42 °C, the growth rate of YRL 005 in YPX was close to that of YPD (Fig. S1A). The expressed XI was determined by the measurement of XI activity. The XI activities in *K. marxianus* YHJ 010, YRL 002, YRL003, and YRL 005 were 0.0017, 0.0013, 0.056, and 0.25 U/mg after 48 h cultivation, respectively. These XI activities were consistent with the growth of each strain. Because YHJ 010 grew well with xylose due to its complete xylose-reductase-xylitol-dehydrogenase pathway, its XI activity was very weak, which could be considered as background. In YRL 003, the XI activity increased dozens of times compared to YHJ 010 and YRL 002, but it was not strong enough to support its growth with xylose as the carbon source. Moreover, in YRL 005, the XI activity increased 4.5-fold further, sufficient to support growth on xylose. In fact, the adaptation procedure was an XI activity-increasing procedure. To figure out the reason that the XI expression increased, YRL 005 was cultured in YPX at 42 °C and its genomic DNA was extracted. The *XylA* gene was amplified together with the GAPDH promoter with the primers GAP-StuI-F and XI-NotI-R (Table 1) and validated by sequencing. No mutation was found in the GAPDH promoter and *XylA* region, so the increase of expression should be due to the mutant(s) of other gene(s) or the changed expression of other gene(s). Real-time PCR was used to analyze the related gene expression, such as *xylA*, *Kmxyl3*, *Kmtal1*, *Kmtkl1*, *Kmrpe1*, and *Kmrki1*. The cells of YRL 003 and YRL 005 were cultivated in YPD medium at 42 °C for 12 h in which exponential phase as pre-culture and trans-inoculated into YPX culture and cultivated for another 12 h for total RNA extraction. Compared with internal control *act1* and *gapdh*, the expression level of *Kmxyl3*, *Kmtal1*, *Kmtkl1*, *Kmrpe1*, and *Kmrki1* were increased greatly after adaptation in YRL 005 (Table 4). Before adaptation, the expressions of the above genes were from 0.11 to 2.52-fold of internal control. However, after adaptation, the expressions of these genes were 1.77–11.08-fold of internal control (Table 4). The expression of *xylA* was similar before and after adaptation (2.36 and 2.05-fold of *act1*, respectively), but the activity of *xylA* increased greatly and the values were 0.056 U/mg and 0.25 U/mg before and after adaptation. It is possible that after adaptation the yeast can assimilate xylose and propagate quickly. Therefore, the expressions of a lot of genes including internal control were increased [35] and the absolute expression level of *xylA* also

Table 4 Relative expression level of xylose assimilation genes by real-time PCR

Gene	YRL 003		YRL 005	
	actin	*gapdh*	*actin*	*gapdh*[a]
xylA	2.36 ± 0.45	2.52 ± 0.48	2.05 ± 0.46	2.33 ± 0.52
Kmxyl3	0.11 ± 0.022	0.12 ± 0.023	1.77 ± 0.15	2.00 ± 0.16
Kmtkl1	0.96 ± 0.041	1.02 ± 0.044	7.46 ± 0.09	8.45 ± 0.10
Kmtal1	0.97 ± 0.085	1.03 ± 0.090	9.78 ± 1.12	11.08 ± 1.27
Kmrki1	0.24 ± 0.0057	0.26 ± 0.0061	4.40 ± 1.11	4.99 ± 1.26
Kmrpe1	1.84 ± 0.14	1.95 ± 0.15	9.16 ± 2.92	10.38 ± 3.31

[a] This row indicates the internal control used

increased significantly in YRL 005. It could be concluded that the expressions of the above genes were increased after adaptation. As a result, YRL 005 can grow better than YRL003. Though the adaptations at higher temperature such as at 42, 45, and 48 °C were also conducted, no fast xylose assimilation strain was obtained.

After adaptation, YRL 005, which harbored *XylA,* could consume xylose at 0.066 g xylose/g biomass/h in YPX medium under aerobic condition, no xylitol accumulation was detected and ethanol was only detected in 24 h (0.23 g/l) and 30 h (0.41 g/l). Though YHJ 010 could also grow well in YPX medium and consume xylose at a similar rate (0.058 g xylose/g biomass/h), xylitol was accumulated (1.67 g/l) (Fig. 1c). Therefore, in YRL 005, the xylose reductase and xylitol dehydrogenase pathway was successfully substituted by the isomerase pathway.

The growth of each strain was also determined at 45 °C with YPX. *K. marxianus* YHJ 010 was also found to grow well, reaching an OD_{600} of 9.12 in 30 h. However, prior to 48 h, the growth of YRL 005 was weak. After 48-h cultivation, YRL 005 began to grow, and it reached an OD_{600} of 9.01 after another 24 h of culture (Fig. 1d). To find out the reason for the delay, the transcriptional level and XI activity during the culture were determined. The XI activities of *K. marxianus* YRL 005 in the lag phase (24 h), exponential phase (52 and 58 h) and stationary phase (96 h) were measured (Table 5). They were 0.028, 0.15, 0.21, and 0.16 U/mg, respectively. The transcriptional level of *XylA* in the lag phase (24 h), exponential phase (52 and 58 h) and stationary phase (96 h) were also determined through real-time PCR analysis (Table 5). In the lag phase (24 h), the relative transcriptional level of *xylA* was a little weaker than the internal control (only 0.88 and 0.75-fold of *act1* and *gapdh*, respectively). However, during the exponential phase (52 and 58 h), the relative transcriptional level increased significantly (3.84 to 3.56-fold of *act1* and 2.89 to 2.79-fold of *gapdh,* respectively). In the stationary phase, though the expression of *xylA* might decrease, they were still 2.27-fold of *act1* and 1.97-fold of *gapdh* (Table 5). The changes of XI activity and transcriptional level according to the growth curve imply that the delay was possibly due to the delayed XI expression. This

Table 5 *XylA* expressional and XI activity of YRL 005 cultivated with YPX at 45 °C

Time	XI activity (U/mg)	Relative expression	
		actin	*gapdh*[a]
Lag phase (24 h)	0.028 ± 0.002	0.88 ± 0.08	0.75 ± 0.07
Exponential phase (52 h)	0.15 ± 0.02	3.84 ± 0.36	2.89 ± 0.31
Exponential phase (58 h)	0.21 ± 0.03	3.56 ± 0.65	2.79 ± 0.49
Stationary phase (96 h)	0.16 ± 0.009	2.27 ± 0.24	1.97 ± 0.29

[a] This row indicates the internal control used

phenomenon was also detected in GAPDH promoter-controlled *P. stipitis* XR expression in *K. marxianus* when an XR-substituted strain was constructed [49]. It is possible that the delay of XI expression was due to the transcription controlled by the GAPDH promoter at 45 °C. Kondo's group also reported that the expression of lipase is weaker at a higher temperature under control of the GAPDH promoter [27], but this hypothesis required additional investigations of the promoter.

Fermentation with xylose as carbon source

To determine their xylose fermentation abilities, *K. marxianus* YHJ 010, YRL 002, YRL 003, and YRL 005 were all anaerobically cultivated in sealed anaerobic bottles using YPX at 42 °C as described in the Materials and methods section. YRL 002 and YRL 003 could scarcely assimilate xylose, and they did not produce ethanol (Fig. 2a). Although *K. marxianus* YHJ 010 assimilated xylose well under aerobic conditions (Fig. 1c), the consumption of xylose under anaerobic conditions was greatly decreased (1.47 g/l with 1.2 g/l xylitol produced), and no ethanol production was detected (Fig. 2a). This result is consistent with previous reports that under oxygen-limited conditions, xylose assimilation is hindered by a redox imbalance due to the co-enzyme preferences of xylose reductase and xylitol dehydrogenase. *K. marxianus* YRL 005 was found to ferment xylose and produce ethanol at a

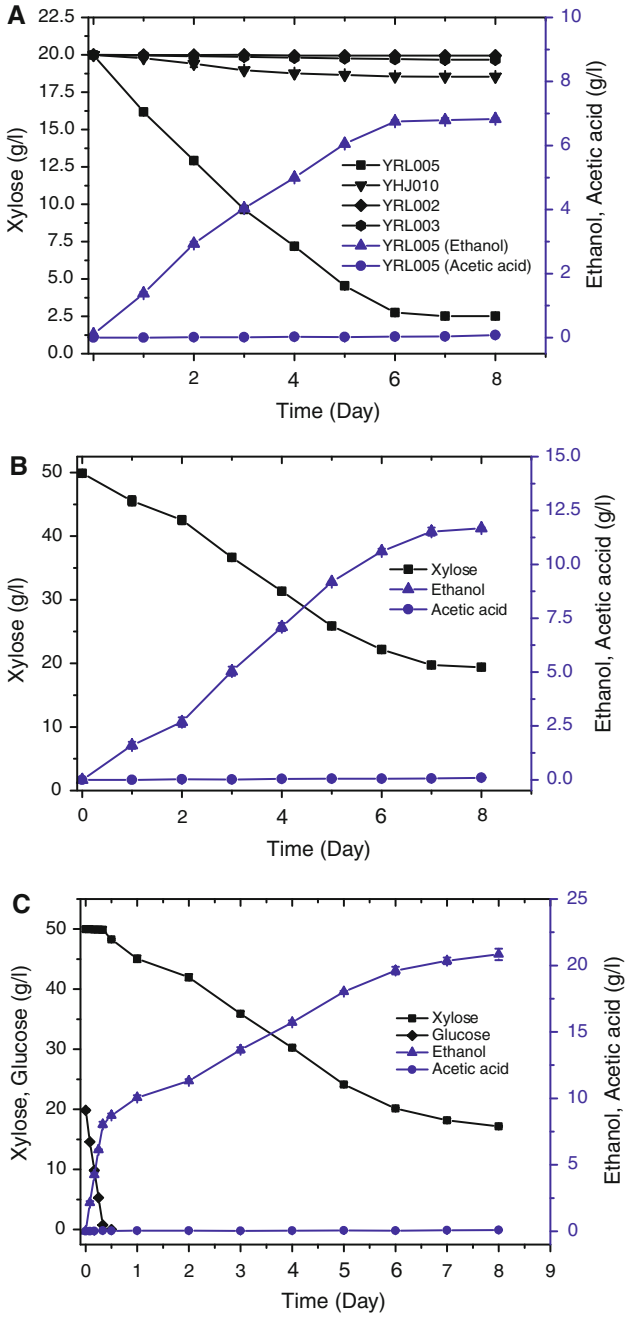

Fig. 2 Fermentation with xylose at 42 °C under anaerobic conditions in YPX. Fermentation of each strain grown with 2 % xylose (**a**), fermentation of *K. marxianus* YRL 005 with 5 % xylose (**b**), or mixture of 2 % glucose and 5 % xylose (**c**). YRL 002: *KmXyl1* and *KmXyl2* double disrupted strain; YRL 003: YRL 002 transformed with the *XylA* gene; YRL 005: strain resulting from adaptation of YRL 003, YHJ 010: *KmTrp1, KmLeu2, KmUra3* auxotrophic strain derived from NBRC 1777

rate of 0.026 g/l/h and produce 6.83 g/l ethanol from 17.474 g/l xylose in 7 days. No xylitol or glycerol accumulation was detected (Fig. 2a). Although acetic acid was detected, the concentration was very low (only 0.036 g/l at the 7th day).

To compare with other *xylA*-expression recombinant yeasts and evaluate the fermentation with higher concentrations of xylose, *K. marxianus* YRL 005 was anaerobically cultivated at 42 °C in YPX with 5 % xylose as the carbon source. Because the strain YRL 002, YRL 003, and YHJ 010 hardly utilized xylose and did not produce ethanol at 42 °C with 2 % xylose, they were not investigated further with 5 % xylose. With the increased xylose concentration, the concentration of ethanol produced by YRL005 increased to 11.52 g/l with 30.15 g/l xylose consumed after 7 days of culture, and the production rate was 0.069 g/l/h (Table 3; Fig. 2b). No xylitol or glycerol accumulation was detected. Similar to the fermentation with 2 % xylose, the concentration of acetic acid produced with 5 % xylose was also low (only 0.063 g/l on the 7th day). Although a higher concentration of xylose was used and more ethanol was produced, the concentration of acetic acid produced was still kept at low concentration.

Co-fermentation of glucose and xylose is advantageous for large-scale ethanol production with sugar from biomass as the growth substrate. Co-fermentation with a 2 % glucose and 5 % xylose mixture was determined at 42 and 45 °C. At 42 °C, 20.35 g/l ethanol was produced after 7 days of fermentation. In the first 8 h, 19.07 g/l glucose was consumed and 8.03 g/l ethanol was produced, comprising approximately 83 % of the theoretical yield (Fig. 2c). However, during this period, xylose was barely consumed. After the glucose was depleted, the xylose began to be assimilated, but its speed was much slower than that of glucose (Fig. 2c). After 7 days of fermentation, 31.10 g/l xylose was consumed and 12.13 g/l ethanol was produced with a production rate of 0.075 g/l/h (Fig. 2c), which was slightly higher than fermentation with xylose alone (0.069 g/l/h). Acetic acid was produced at 8 h, and the concentration remained very low (0.068 g/l on the 7th day); thus, the mixed sugar did not boost acetic acid production. The *K. marxianus* YRL 005 had a diauxic period during which glucose was consumed firstly and quickly when a sugar mixture was used and previous report has proved that xylose metabolic genes expression in *K. marxianus* can be repressed by glucose though the mechanism is not clear [32, 33]. In this study, the delay of xylose utilization when glucose was presented was possibly caused by glucose repression. Although the xylose isomerase was expressed under a constitutive promoter (pGAPDH), the expression of the xylulose kinase and other xylose metabolic genes might still been repressed by glucose. The xylose transport by sugar transporters could also be strongly inhibited by glucose as reported in other yeasts [3]. In order to construct a strain that could consume glucose and xylose simultaneously, genetic modification of the expression of the above genes, such as the modification or change of their promoters or the deletion or attenuation of

Table 6 Comparison of xylose consumption and ethanol formation among recombinant yeast strains with the XI pathway

Xylose (g/l)	Xylose consumption (g/l)	Temperature (°C)	Ethanol yield (g/g)	Ethanol (g/l)	Ethanol productivity (g/l/h)	Time (h)	Yeast	Reference
20	16	30	0.31	4.9	0.02	240	*S. cerevisiae* LBsXI	[11]
50	27.9 ± 0.7	30	0.43 ± 0.07	11.9	0.119	100	*S. cerevisiae* TMB23362	[30]
30	28.91	30	0.347	9.36	0.078	72	*S. cerevisiae* MT8-1ΔGRE3/XKδXI	[42]
25	18	30	0.42	7.6	0.045	170	*S. cerevisiae* BWY10Xyl	[5]
20	14.84	30	0.43	6.32	0.07	90	*S. cerevisiae* ADAP8	[25]
50	15.05	30	0.39	6.05	0.043	140	*S. cerevisiae* ADAP8	[25]
20	20	30	0.43	8.68	0.18	48	*S. cerevisiae* RWB 217	[21]
30	10.4	38	0.12	1.3	0.012	110	*S. cerevisiae* H158(pBXI)	[46]
40	30	0.68	0.41	16.4	0.68	24	*S. cerevisiae* H131-A3-AL^{CS}	[51]
80	NA	37	NA	1.03	0.011	96	*H. polymorpha* $^{\Delta}$xyl1 $^{\Delta}$xyl2-A(EcxylA) #4L/3	[9]
80	NA	48	NA	0.6	0.006	96	*H. polymorpha* $^{\Delta}$xyl1 $^{\Delta}$xyl2-A(EcxylA) #4L/3	[9]
50	11.52 ± 0.19	42	0.38 ± 0.003	11.52 ± 0.019	0.069	168	*K. marxianus* YRL 005	This study
50	5.21 ± 0.07	45	0.31 ± 0.014	16.6 ± 0.11	0.054	96	*K. marxianus* YRL 005	This study

If a reference contained several strains, only the best one is shown here. The data without the standard deviation were due to no standard deviation provided in the original paper

Values are presented as mean and standard deviation of three independent experiments

glucose repressors [31], would be necessary. Engineering of sugar transporters to reduce the inhibition of glucose could be another path.

Similar to the results of some other yeast strains, the xylose was not completely consumed (Table 6; Figs. 2, 3). There were several possible reasons that led to the incomplete consumption. Firstly, the transporter and downstream enzyme activity were not strong enough; secondly, the coenzyme and energy (such as ATP for xylulose kinase and other enzymes) were limited after anaerobic culture with xylose, and finally, the accumulation of metabolic product that could inhibit the fermentation at very low concentration was also a possible reason. However, more research is needed to confirm these possible explanations.

As *K. marxianus* can grow and produce ethanol well from glucose at 45 °C [14], fermentation of *K. marxianus* YRL 005 with 20 g/l xylose was also performed at 45 °C and the ethanol was produced at a rate of 0.031 g/l/h and only 2.27 g/l ethanol with 7.25 g/l xylose consumed in 3 days. No xylitol or glycerol accumulation was detected, but 0.69 g/l xylulose was accumulated and 0.013 g/l acetic acid was detected, at the third day. Fermentation was also conducted with 50 g/l xylose. Similar to most previous reports (Table 3), the ethanol yield and the amount of ethanol produced from 50 g/l xylose at 45 °C (0.31 g/g, 5.21 g/l) was lower than that at 42 °C (0.38 g/g, 11.52 g/l). However, compared with other *K. marxianus* strains, more ethanol was produced at 45 °C, and the fermentation was only detected during the first 4 days. A total of 16.60 g/l xylose was consumed and 5.21 g/l ethanol was formed with a yield of 0.31 g/g during this period. The acetic acid formation was similar to that at 42 °C (0.037 g/l on the 5th day) and xylulose was also accumulated (1.95 g/l) (Fig. 3a). The co-fermentation showed that the fermentation was also nearly completed after 4 days. During this period, 19.85 g/l glucose and 17.27 g/l xylose were consumed and 13.52 g/l ethanol was produced (Fig. 3b). A total of 19.85 g/l glucose was consumed over 8 h and produced 7.97 g/l ethanol with a yield of 0.40 g/g, which was similar to the fermentation at 42 °C. After the glucose was depleted, 17.27 g/l xylose was consumed and 5.56 g/l ethanol was produced with a yield of 0.31 g/g, which was almost the same as for xylose only. However, the production rate (0.064 g/l/h) was higher than for xylose (0.054 g/l/h) (Fig. 3b). To ascertain why the fermentation stopped after 4 days, the thermo-stability of crude XI was determined. After incubation for 10 min at 37, 42, and 45 °C, the retained relative activity was 95.75, 83.50, and 67.47 %, respectively. Therefore, the XI expressed was not thermo-stable. The XI activity in YRL 005 cells cultivated at 37 and 45 °C were 0.25 and 0.18 U/mg, respectively. The decreased expression and instability of XI at 45 °C are possibly the reasons that the fermentations stopped. Xylulose accumulation was also detected in the co-fermentation. This result may possibly be due to the activity of an

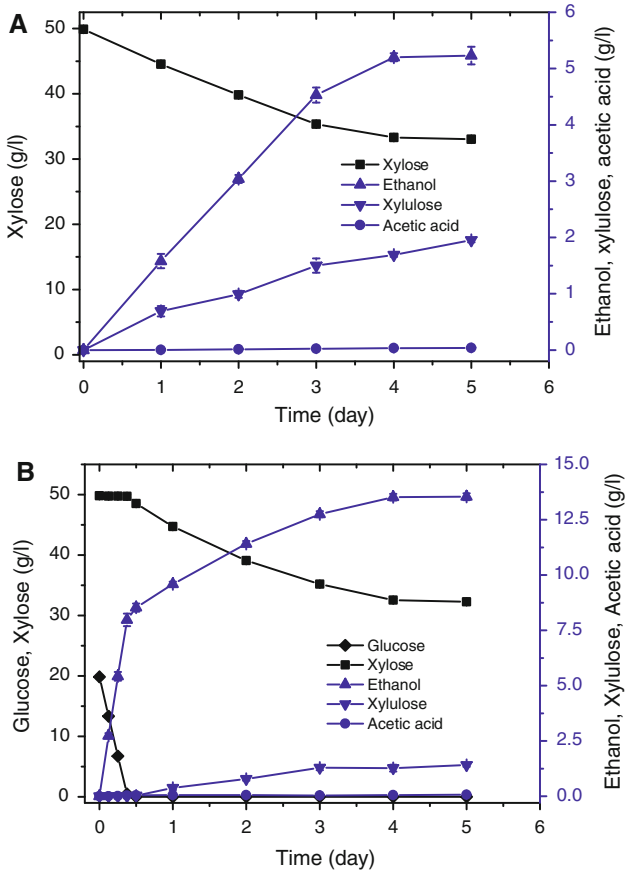

Fig. 3 Fermentation of *K. marxianus* YRL 005 with xylose at 45 °C under anaerobic conditions in YPX. Xylose consumption, xylitol accumulation, xylulose accumulation, and ethanol production with 5 % xylose (**a**) or 2 % glucose and 5 % xylose (**b**)

XK that was not strong enough or a downstream metabolic rate that was not fast enough under anaerobic conditions at 45 °C.

Although there was no xylose reductase expressed when the XI pathway was constructed in *S. cerevisiae*, xylitol could nonetheless be accumulated due to the non-specific aldose reductase GRE3 [43]. In YRL 005, there was no detectable xylitol accumulation. This was possibly due to two reasons: no aldose reductase similar to GRE3 may have existed or the downstream catabolic rate may have been faster than the xylulose formation. Nonetheless, at 45 °C, even though xylulose was accumulated, there was still no xylitol formed. Therefore, nonexistence of a GRE3-like aldose reductase was most likely the main reason.

Xylose fermentation with *S. cerevisiae* strains through an engineered xylose reductase and xylitol dehydrogenase pathway could produce more ethanol such as approximately 28 g/l ethanol by strain TMB3367, a better performance than other yeast strains through XI pathway (Table 6) [30]. This result is due to the extensive genetic modification required to modify the recombinant strains. In the recombinant reductase-dehydrogenase-pathway in *S. cerevisiae*, numerous improvements, including the engineering of sugar transport [19], initial conversion of the pentose sugars [44], enhancement of the pentose phosphate pathway (PPP) [17, 18], and the modification of redox cofactor utilization [16, 47], were conducted. However, the successful cases of isomerase engineering are few, and include less metabolic optimization work. Recently, a *S. cerevisiae* strain expressing the *Piromyces XylA*, *P. stipitis* xylulose kinase (*Xyl3*) and the genes of the non-oxidative PPP was constructed. With this strain, 16.4 g/l ethanol were produced from 40 g/l xylose [51]. An isomerase pathway that can avoid the redox imbalance under oxygen-limited conditions and that has a higher conversion rate was thought to have a better future if the xylose fermentation-related genes were carefully regulated.

In this study, with 20 g/l xylose, 6.74 g/l of ethanol was produced, which was more than threefold of other reported *K. marxianus* strains at 42 °C. With 50 g/l xylose, 11.52 g/l ethanol was produced, which was the highest in the yeasts at elevated temperature (Table 6). The yields were 0.39 g/g with 20 g/l xylose and 0.38 g/g with 50 g/l xylose, respectively, also the highest reported for a *K. marxianus* strain (Table 3).

Fermentation with corn-cob hydrolysate

Corn cob hydrolysate was used to evaluate the fermentation ability of *K. marxianus* YRL 005 (Fig. 4). The prepared corn cob hydrolysate contained 23.61 g/l xylose, 1.62 g/l glucose, 0.96 g/l fructose, and 2.17 g/l arabinose, and no furfural was detected. At 42 °C, the fermentation was similar to that with pure xylose (8.25 g/l ethanol produced from the hydrolysate with 20.04 g/l xylose in addition to the glucose and fructose). At 45 °C, the fermentation was weak and only 2.88 g/l ethanol was produced with only 6.14 g/l xylose consumed in the first 3 days, then the xylose consumption nearly stopped.

In fact, in the commercial application of xylose fermentation, pure xylose would not be used; instead, other materials such as xylose in biomass hydrolysates are more likely to be used. Here, the hydrolysate from corn cob was used as an example. Dilute-acid pretreatment can result in high xylose release, converted from xylan. The xylose derived from lignocellulosic biomass such as corn cob is a valuable substrate for xylitol and ethanol production [22].

Conclusions

In this study, a xylose metabolic pathway through xylose isomerase was constructed in thermo-tolerant yeast

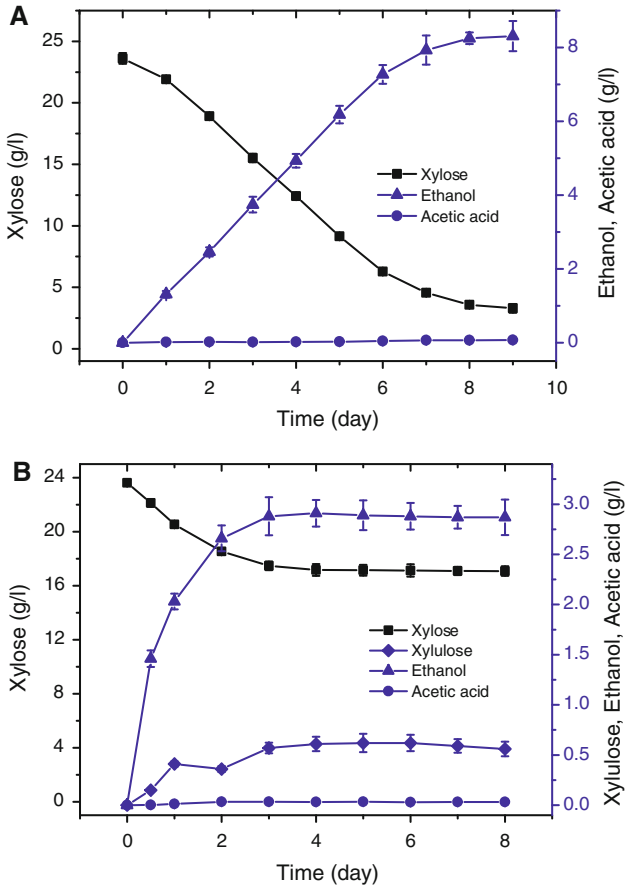

Fig. 4 Anaerobic fermentation with corn-cob hydrolysate in YP medium using *K. marxianus* YRL 005 at 42 °C (**a**) and at 45 °C (**b**)

K. marxianus YRL 005. The constructed recombinant yeast could ferment xylose to produce ethanol at temperatures above 42 °C, with no detectable xylitol, glycerol, and only very small amounts of byproducts, such as acetic acid, produced. The ethanol production rate and xylose consumption rate of YRL 005 under anaerobic conditions are best in those strains that ferment xylose at temperatures above 42 °C. Therefore, the YRL 005 provides an excellent platform for improving xylose fermentation at elevated temperatures.

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Conflict of interest The authors have no conflict of interest to report.

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