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ORIGINAL PAPER

Identification of a xylose reductase gene in the xylose metabolic pathway of *Kluyveromyces marxianus* NBRC1777

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Abstract *Kluyveromyces marxianus* is thermotolerant yeast that is able to utilize a wider range of substrates and has greater thermal tolerance than most other yeast species. *K. marxianus* can assimilate xylose, but its ability to produce ethanol from xylose in oxygen-limited environments is poor. In the present study, the *K. marxianus* xylose reductase (KmXR) gene (*Kmxyl1*) was cloned and the recombinant enzyme was characterized to clarify the factors that limit xylose fermentation in *K. marxianus* NBRC1777. KmXR is a key enzyme in the xylose metabolism of *K. marxianus*, which was verified by disruption of the *Kmxyl1* gene. The *Km* of the recombinant KmXR for NADPH is 65.67 μM and KmXR activity is 1.295 U/mg, which is lower than those of most reported yeast XRs, and the enzyme has no activity with coenzyme NADH. This result demonstrates that the XR from *K. marxianus* is highly coenzyme specific; combined with the extremely low XDH activity of *K. marxianus* with $NADP^+$, the limitation of xylose fermentation is due to a redox imbalance under anaerobic conditions and low KmXR activity.

Keywords Xylose reductase · *Kluyveromyces marxianus* · Xylose utilization · Coenzyme specificity

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Introduction

D-Xylose is one of the main hydrolysis products of lignocellulosic biomass and is the second most abundant fermentable material [6, 19, 33, 38]. In contrast to the efficient glucose fermentation in yeast, xylose fermentation is challenging because only a few ethanol-producing microorganisms can readily ferment xylose even though many microorganisms utilize xylose as a carbon source [19].

Highly efficient xylose fermentation could improve the utilization of biomass [16]. 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 [29, 32]. In bacteria, the D-xylose is converted to D-xylulose directly by xylose isomerase (XI); in eukaryotes such as yeasts and filamentous fungi, D-xylose is converted to D-xylulose through reduction and oxidation, which is catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively [16].

XR is the first enzyme in xylose metabolism and it catalyzes xylose to xylitol in eukaryotes. Generally, poor ethanol production from xylose in yeasts is due to the accumulation of xylitol and the inability to catalyze xylitol to xylulose further during anaerobic fermentation [4]. The main limiting factor is the imbalance of redox, which is

caused by the difference in the preferred coenzyme of XR (NADPH) and XDH (NAD^+) [37].

K. marxianus can grow at temperatures as high as 52°C [2]. Even at 45°C, *K. marxianus* still can produce ethanol well [14, 22, 23]. *K. marxianus* strains are able to utilize a wider range of substrates and exhibit greater thermal tolerance than most other yeast [28]. One of the important potential applications of these strains is the simultaneous saccharification and fermentation (SSF) of cellulosic biomass [5, 8]. The optimal temperature of cellulases used in SSF is 45–50°C; although most yeast cannot survive at such temperatures, *K. marxianus* strains tolerate higher SSF temperatures. The high fermentation temperature allows more rapid and efficient enzymatic cellulose hydrolysis [3, 34]. Some *K. marxianus* strains are able to consume xylose and produce ethanol in oxygen-limited environments. *K. marxianus* IMB4 produces ethanol from xylose with 23% of the theoretical yield at 45°C [2] and 2.08 g/l ethanol at 40°C [34]. Other *K. marxianus* strains can produce 5.6 g/l ethanol from 20 g/l xylose at 35°C or produce 0.8–1.2 g/l ethanol with 10 g/l xylose [2, 4]. However, these results show that the xylose fermenting ability of *K. marxianus* is not high [2, 4, 21, 34]. To lay the foundation for the further improvement of xylose fermentation in *K. marxianus*, the factors that limit fermentation in this yeast need to be determined.

In the present study, we cloned the *K. marxianus* XR gene (KmXR; *Kmxyl1*) and characterized the recombinant enzyme of *K. marxianus* NBRC1777. The function of the *Kmxyl1* in the xylose metabolism of *K. marxianus* was confirmed by disruption of *xyl1* in the genome. Combined with the properties of XDH in the yeast, we confirmed that coenzyme bias and low activity were the main reasons that hinder efficient xylose fermentation in *K. marxianus*.

Materials and methods

Reagents and microorganisms

All chemicals used were of analytical grade or higher. D-Xylose and dNTPs were obtained from Sangon Biotech Co. (Shanghai, China); D-galactose, D-arabinose, D-mannose, Coomassie Brilliant Blue R-250, NADH, NADPH, and agar A were obtained from Bio Basic, Inc. (Toronto, Canada). Restriction enzymes and modifying enzymes were obtained from Fermentas Life Sciences (Fermentas China, Shengzheng, China). The yeast extract and peptone were purchased from Oxoid (Netherlands). *K. marxianus* NBRC1777 was from NBRC (Tokyo, Japan). *K. marxianus* strains were cultured in yeast extract/peptone-dextrose (YPD) medium. *Escherichia coli* XL10-Gold and BL21 star (DE3) were used for cloning and expression, respectively, and were grown in Luria–Bertani (LB) medium.

Cloning of *Kmxyl1* gene from *K. marxianus* NBRC1777

K. marxianus NBRC1777 was cultured in YPD medium at 37°C for 24 h, after which the genomic DNA was extracted, as described before, with glass beads and vortex to break the yeast cell [15]. The part sequence of the XR gene (*Kmxyl1*) was amplified by PCR with a pair of degenerated primers (XR-F1 and XR-R1 in Table 1), which were designed based on conserved amino acid sequence of XR (EKYPPGFY and RFNDPWD/EW) (Fig. 1).

After getting the partial sequence of *Kmxyl1*, the unknown flanking sequences were amplified by thermal asymmetric interlaced PCR (TAIL-PCR) [20]. The specific primers for the 3′ terminal and 5′ flanking sequence were XR-FS1, XR-FS2, XR-FS3 and XR-RS1, XR-RS2, XR-RS3, respectively. The arbitrary degenerate (AD) primers were AD1, AD2, AD3, and AD4. The arbitrary primer for the second and third round TAIL-PCR was AC1 (Table 1). The PCR cycle conditions were the same as those described by Liu [20]. Amplified flanking sequences were cloned into pMD18-T and validated by sequencing. The full-length sequence of *Kmxyl1* was obtained through the assembly all the sequences.

Protein expression and purification

The ORF of *Kmxyl1* was amplified by PCR with primers XR-NDE and XR-HIND (Table 1) from the genomic DNA of *K. marxianus* NBRC1777. The PCR products and plasmid pET-21c were double digested with *Nde* I and *Hin*d III, and then ligated to construct plasmid pET-21XR. This plasmid was transformed into *E. coli* BL21 (DE3). One of the positive colonies was inoculated into liquid LB medium that contained 100 μg/ml ampicillin and was cultured at 37°C. When OD_{600} reached 0.4, isopropyl-α-D-thiogalactopyranoside (IPTG) was added with a final concentration of 0.02 mM and incubation was continued for 4 h. Subsequently, the cells were harvested and resuspended in Tris–HCl buffer (50 mM Tris–HCl and 25 mM sodium chloride at pH 8.0). Furthermore, the cells were lysed by sonication (Vibra-Cell VC505, Connecticut, USA) for 8 min at 40% power. The supernate and lysate pellet fractions were separated by centrifugation at 7,000 × *g* for 30 min. Afterwards, the recovered supernate was loaded onto a Ni^{2+}-chelating column (Qiagen, USA) and washed according to the manual. Subsequently, the purified proteins were eluted from the column by a gradient of 1 to 250 mM imidazole in 50 mM Tris–HCl, 300 mM sodium chloride buffer (pH 8.0). All samples from the column were analyzed by SDS-PAGE and the purified recombinant KmXR was desalted by dialyzing against 100 mM phosphate buffer (pH 8.0). The purified protein was stored with 20% glycerol at –80°C. Protein concentrations were determined via the Bradford method with bovine serum albumin (BSA) as the protein standard [9].

Table 1 Primers used in the present study

Primer	Sequence
XR-F1	5′-GARAARTAYCCWCCWGGWTTYTA-3′
XR-R1	5′-CCAKTCCCAWGGRTCRTTGAATC-3′
AD1	5′-ACGATGGACTCCAGAGAVNVNNNGGAA-3′
AD2	5′-ACGATGGACTCCAGAGABNBNNNGGTT-3′
AD3	5′-ACGATGGACTCCAGAGVVNVNNNCCAA-3′
AD4	5′-ACGATGGACTCCAGAGBBNBNNNCGGT-3′
AC1	5′-ACGATGGACTCCAGAG-3′
XR-FS1	5′-CGCCTACTCCAGTTTCGGCCCACTATCCTT-3′
XR-FS2	5′-GCCGTCATTCCAAAGTCCTC-3′
XR-FS3	5′-GTCACCCCATCCCACGTCTTGT-3′
XR-RS1	5′-TTCTTGGAGGACTTTGGAATGACGGCGATA-3′
XR-RS2	5-′CCAACTTCTCAAGGGCTCTGT-3′
XR-RS3	5′-ACTGGAGTAGGCGACGACTTGC-3′
XR-NDE	5′-GGGAATTCCATATGACATACCTCGCACCAACA-3′
XR-HIND	5′-CCCAAGCTTGATAAAGGTTGGGAATTCG-3′
TRP-SAL-F	5′-ACGCGTCGACACTCTTCCTTTTTCAATATTA-3′
TRP-SAL-R	5′-ACGCGTCGACTTATCCCATTCCATGCGGGGT-3′
XR-F	5′-ATGACATACCTCGCACCAACA-3′
XR-R	5′-TTAGATAAAGGTTGGGAATTCG-3′
STOD-F	5′-CGTGGTATCGCCGTCATTCCAAAGTCCGATAAGAAGGAACGTCTCCTCGA-3′
STOD-R	5′-TCGAGGAGACGTTCCTTCTTATCGGACTTTGGAATGACGGCGATACCACG-3′
STON-F	5′-CGTGGTATCGCCGTCATTCCAAAGTCCAACAAGAAGGAACGTCTCCTCGA-3′
STOD-R	5′-TCGAGGAGACGTTCCTTCTTGTTGGACTTTGGAATGACGGCGATACCACG-3′

Underlined, the restricted enzyme site

Codes for degenerate bases in primers: K (G or T), Y(C or T), R (A or G), W (A or T), B (C, G, or T), V (A, C, or G), D (A, G, or T), N (A, C, G, or T)

XR activity assay

The activity of KmXR was determined according to a previously described method [36, 38] using a spectrophotometer to monitor the change in A_{340} upon oxidation of NAD(P)H. Unless indicated otherwise, the KmXR assay mixture (1.0 ml) for the reaction contained 100 mM phosphate buffer (pH 7.0), 200 μM NAD(P)H, 200 mM xylose, and enzyme solution (0.1 ml). This reaction mixture was allowed to stand for 1 min to eliminate the endogenous oxidation of NADPH, and the reaction was started by the addition of 0.1 ml of substrate. 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.

Optimal temperature and thermostability

The optimal temperature was studied by measuring KmXR activity at temperatures ranging from 15 to 40°C. To determine the thermal stability of KmXR, the enzyme was incubated at 30–60°C for 10 min. The retained enzyme activity was measured as described in the XR activity assay.

Optimal pH

To determine the optimal pH, enzyme activity was measured at pH levels from 5.0 to 9.0. The following buffers were used to adjust the pH of the reaction mixture: 100 mM acetic acid–sodium acetate buffer (for pH 5.0–5.5), 100 mM phosphate buffer (for pH 6.0–7.5), and 100 mM Tris–HCl buffer (for pH 8.0–9.0).

Substrate specificity

The substrate specificity of KmXR was measured by replacing 200 mM D-xylose with 200 mM D-glucose, D-ribose, D-galactose, or D-arabinose as the substrate in the reaction mixture. The other conditions were as described in the KmXR activity assay.

Effects of metal ion and DTT

The purified recombinant KmXR was dialyzed in 100 mM phosphate buffer (pH 7.0) containing 10 mM EDTA at 4°C overnight to remove the metal ions, after which the enzyme was dialyzed in 100 mM phosphate buffer (pH 7.0, EDTA-free) for 9 h at 4°C to remove EDTA. Thereafter, the activity

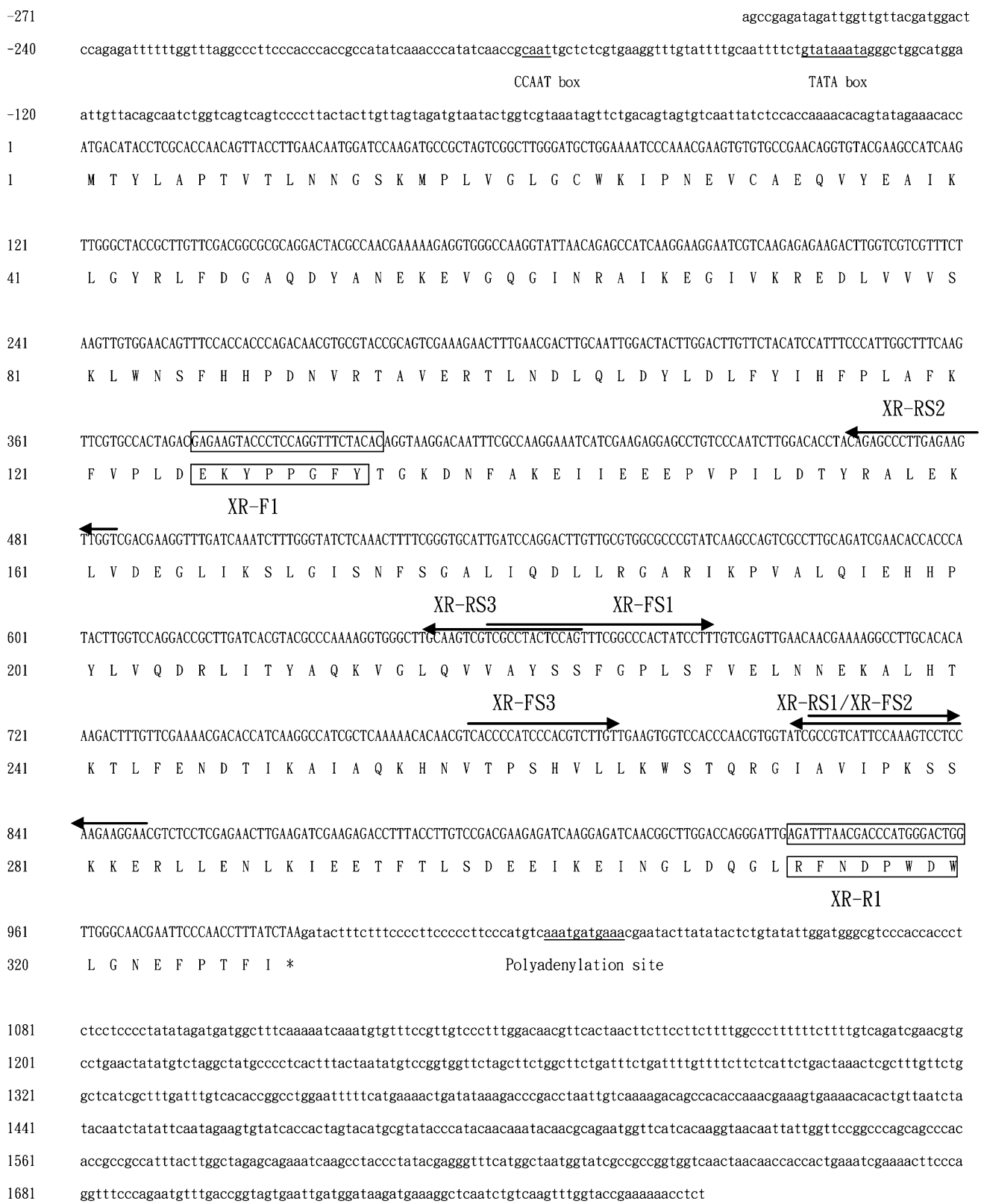

Fig. 1 Nucleotide sequence of the *xyl1* gene from *K. marxianus* and its deduced amino acid sequence. The untranslated and coding regions are represented by *small letters* and *capital letters*, respectively, and the *asterisk* indicates the stop codon. Underlined nucleotides represent the proposed TATA *box* in the 5′ region and the polyadenylation site in the 3′ region. *Arrows* indicate the annealing position and direction of primer extension for TAIL-PCR; the *open box* shows the position on which the degenerated primers design is based

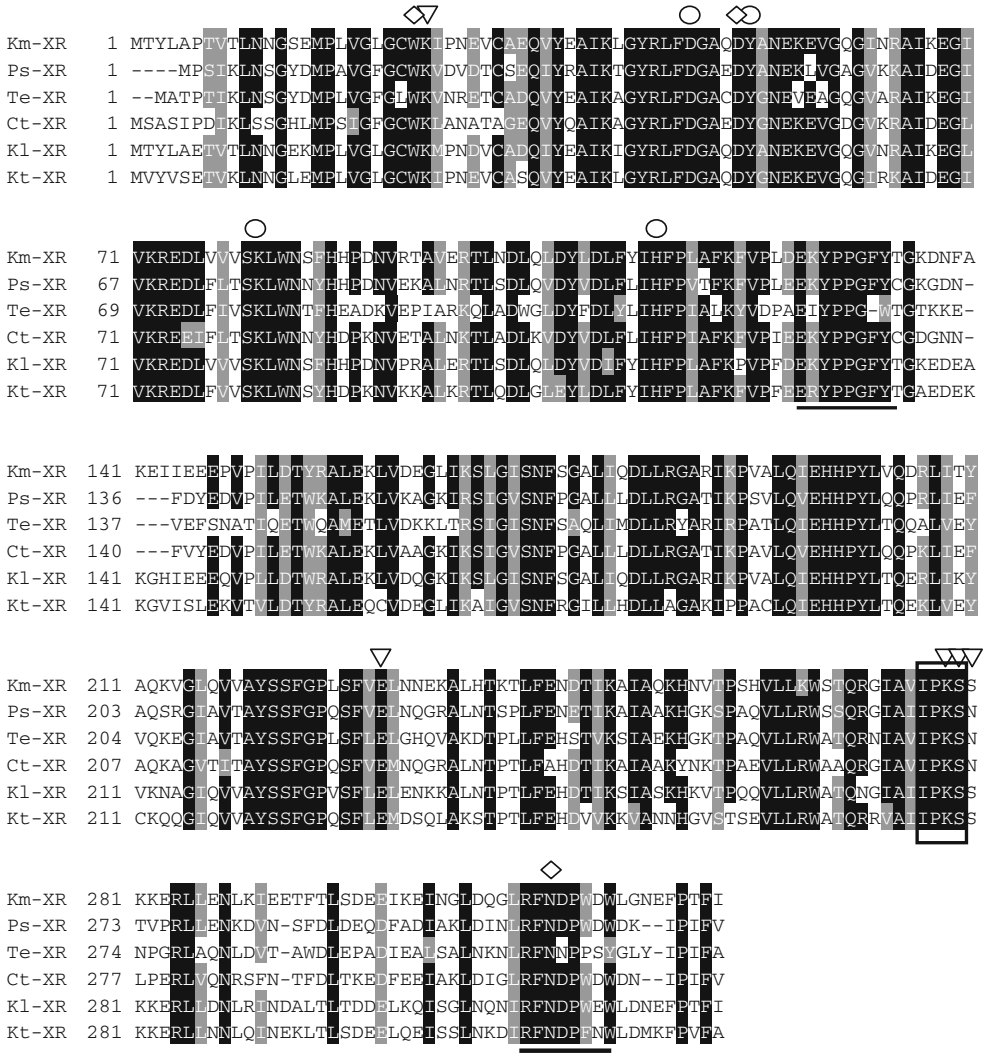

Fig. 2 Alignment of KmXR amino acid sequences with xylose reductases from other yeast species. KmXR, GenBank accession GU574744; PsXR from *Pichia stipitis*, GenBank accession no. CAA42072, 63% identity; CtXR from *Candida tenuis*, GenBank accession no. AAC25601, 60% identity; Te-XR: *Talaromyces emersonii,* GenBank accession no. ACR78268, 55%; KlXR from *K. lactis*, GenBank accession no. AAA99507.1, 79% identity; KtXR from *K. thermotolerans*, GenBank accession no. CAR24470.1, 67% identity. The underlined conserved sequences were used to design degenerated primers for the *Kmxyl1* cloning. The active site (*open circle*), substrate-binding residues (*open diamond*), coenzyme utilization (*open inverted triangle*) are shown, the Ile-Pro-Lys-Ser motif is enclosed by a *rectangle box*

of the recombinant KmXR was measured in the presence of 1 mM CuSO4, 1 mM $MnCl_2$, 1 mM MgSO4, 1 mM $CoCl_2$, 1 mM $NiSO_4$, 1 mM $ZnCl_2$, 1 mM $FeCl_3$, 1 mM $CaCl_2$, or 1 mM DTT in the reaction mixture [100 mM phosphate buffer (pH 7.0), 0.02 mM NADPH, 200 mM xylose].

Kinetics assay

The kinetic parameters for xylose were obtained from the initial velocity measured at a constant NADPH concentration (0.02 mM) with xylose of various concentrations. The kinetic parameters for NADPH were determined using a constant xylose concentration (0.2 M) and varying NADPH concentrations. All measurements were performed at optimal pH and optimal temperature. All results were performed in triplicate and expressed as mean values; the bars in the figures indicate the ranges of the errors.

Kmxyl1 disruption

Homologous recombination [1, 10] was used to disrupt *Kmxyl1* in *K. marxianus* YHJ010 [15], which is the *ura3, trp1*, and *leu2* auxotroph strain from *K. marxianus* NBRC1777. The *trp1* gene of *Saccharomyces cerevisiae* was amplified from the plasmid YEGAP [13] with the primers TRP-SAL-F/TRP-SAL-R (Table 1). The amplified *trp1* and pET21XR were digested with *Sal*I and ligated together to produce pET21XR-TRP. The *Kmxyl1* gene with

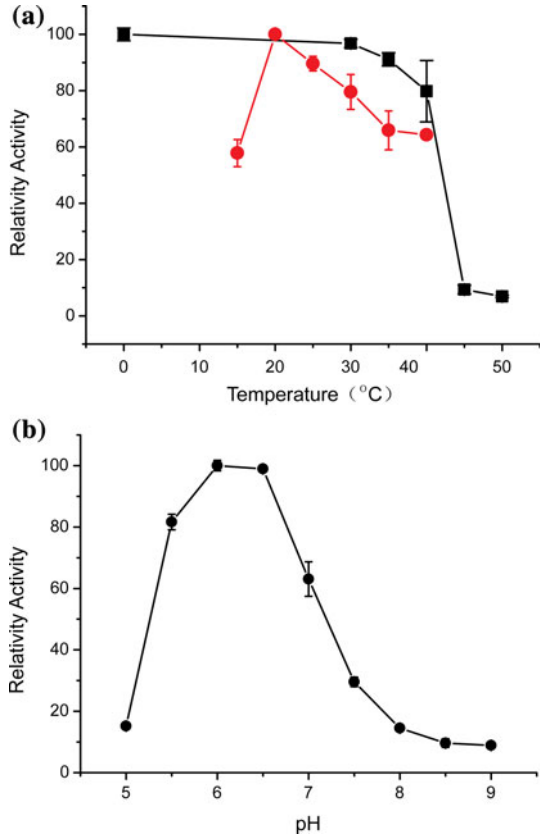

Fig. 3 The activity of XR was determined with 0.2 M xylose and 0.2 mM NADPH. **a** Thermal profile. The optimal temperature (*filled circle*) was determined by assaying XR at pH 7.0 with different temperatures, ranging from 15 to 40°C. The thermostability assay (*filled square*) of XR was taken by treating the enzyme for 10 min at 30–50°C. **b** Profile of pH effects on XR activity

inserted *trp1* was amplified by PCR using the primers XR-F/XR-R (Table 1) and transformed into *K. marxianus* YHJ010. The positive colonies were screened on synthetic dextrose (SD) medium containing uracil and leucine. The obtained colonies were further selected in the SD medium with D-glucose, D-xylose (SDX1), or D-xylitol (SDX2) as the sole carbon sources.

Site-directed mutagenesis of the Ser-280 in KmXR

From the alignment of xylose reductase amino acid sequence and the mutation results reported by other research, Ser-280 in KmXR which is Asn at this position in most XR reported was estimated to be the amino acid residue leading to the relativity low activity and coenzyme specific of KmXR. Site-directed mutagenesis was used to verify its function. The mutations were constructed with overlap extension PCR with the primers STOD-F/STOD-R (for S280D) and STON-F/STON-R (for S280 N) plus the full-length primers XR-NDE and XR-HIND. S280D and S280 N mutants of KmXR was expressed in *E. coli* and characterized.

Table 2 The effects of chemicals

Chemical substance	Relative activity (%)
No ion	100.00 ± 1.62
Mn^{2+}	107.79 ± 10.79
Co^{2+}	109.03 ± 1.94
Mg^{2+}	107.48 ± 5.61
Ca^{2+}	127.10 ± 7.42
Ni^{2+}	104.36 ± 2.16
Zn^{2+}	161.06 ± 24.38
Fe^{3+}	0
Cu^{2+}	0
DTT	143.92 ± 4.28

Table 3 Subtract specificity of *K. marxianus* xylose reductase

Subtract	Relative activity (%)
D-Xylose	100.00 ± 1.76
D-Galactose	75.77 ± 1.66
D-Ribose	66.92 ± 2.31
D-Arabinose	58.65 ± 2.33
D-Glucose	46.92 ± 0.33

Results and discussion

Cloning and sequence analysis of *Kmxyl1* gene from *K. marxianus* NBRC1777

The *K. marxianus* genome is currently being sequenced by both the Institut Pasteur (Genolevures) and NITE, but the projects are listed as incomplete [http://genomesonline.org/cgi-bin/GOLD/bin/Search.cgi], thus, the *Kmxyl1* gene was cloned using degenerate PCR amplification. The full-length sequence of KmXR (2,036 bp), including the complete ORF (from position 272 to 1,261, total 990 bp), was obtained (GenBank accession No. GU574744) via TAIL-PCR following the method described in section "Cloning of *Kmxyl1* gene from *K. marxianus* NBRC1777". The potential TATA box in the 5′ region and the polyadenylation site in the 3′ region were found in non-coding regions (Fig. 1). The amino acid sequence of KmXR was highly homologous with other yeast XR (highest was with the XR from

K. lactis at 79% identity). All the active sites, substrate-binding residues, and coenzyme utilization were conserved, except for one coenzyme-binding site (Fig. 2) [11, 17].

Protein expression and purification

After obtaining the full length of gene *Kmxyl1*, the ORF were amplified by PCR, inserted into pET-21c (Merck, Germany), and subsequently transformed into *E. coli* BL21 (DE3) for expression. The mutants S280D and S280 N were also expressed in pET-21c by *E. coli* BL21 (DE3). The recombinant wild-type and mutation KmXRs in the cell lysate were purified by the Ni^{2+}-chelating affinity chromatography and were almost single bands on SDS-PAGE gel, with a molecular mass of around 37 kDa, as calculated theoretically (data not shown).

Characterization of recombinant KmXR

The optimal temperature was studied by measuring XR activity at temperatures ranging from 15–40°C. As shown in Fig. 3a, the optimal temperature was approximately 20°C. To determine the thermal stability, the retained activity of KmXR was measured after incubation for 10 min from 30–50°C. The half-life of KmXR at 42°C was 10 min, whereas the optimal temperature was 20°C (Fig. 3a). The effects of pH on the enzyme activities were determined at pH levels ranging from 5.0–9.0, with the optimal pH at around 6.5 (Fig. 3b).

The effects of metal ions on KmXR activity were also measured. The results showed that Cu^{2+} and Fe^{3+} completely inhibited the activity of KmXR. Zn^{2+} and Ca^{2+} activated the enzyme greatly and reached 161.06 and 127.10% that of activity without ions, respectively. Other ions had no evident enhancing effects. The 1 mM DTT also improved enzyme activity by approximately 44% (Table 2).

Substrate specificity of KmXR was measured with D-xylose, D-glucose, D-ribose, D-galactose, or D-arabinose as substrates. As shown in Table 3, the optimal substrate was D-xylose (100% activity). Other sugars were converted at 46.92% to 75.77% activity compared with xylose.

XR from *K. marxianus* NBRC1777 has high coenzyme specificity

The *Km* and v_{max} values of KmXR for xylose and NADPH were evaluated using the Michaelis–Menten equation. The *Km* of the *K. marxianus* XR for D-xylose (33.32 mM) was comparable with those of various XRs, but the *Km* for NADPH was 65.7 mM, which was higher than those of XRs from other microorganisms. Furthermore, the recombinant KmXR could not use NADH as a coenzyme (Table 4). This result demonstrates that XR from *K. marxianus* NBRC1777 has high coenzyme specificity.

The *K. marxianus* is a thermotolerant yeast used in numerous studies on SSFs at elevated temperatures. *K. marxianus* could not ferment efficiently if xylose, the main product of xylan, is used as the carbon source to produce ethanol. The final concentration of ethanol was only 0.8–5.6 g/l at 35–45°C [2, 4, 34]. Many factors could cause the inefficient xylose fermentation, of which redox imbalance and low enzyme activity in the xylose metabolic pathway are the most likely [7]. In the present study, the XR activity of *K. marxianus* (1.295 U/mg) was lower than some XRs reported [37, 38], but higher than XR in *Debaryomyces hansenii* UFV-170 [26, 27]. XDH from *K. marxianus* NBRC1777 preferred NAD^+ as the coenzyme and the activity was very low with $NADP^+$ (data not shown). When the yeast is cultured under oxygen-limited conditions, NADH accumulated and NADPH was depleted. Thus, we deduced that redox imbalance; low activity caused the inefficient xylose fermentation in *K. marxianus* NBRC1777 under

Table 4 Properties of various xylose reductases

Organism	*Km* for xylose (mM)	*kcat* for xylose (min^{-1})	*kcat/Km* for xylose ($min^{-1}mM^{-1}$)	*Km* for NADPH (μM)	*Km* for NADH (μM)
K. marxianus (wild-type)	33.3	34.6	1.0	65.7	ND
K. marxianus (S280 N)	19.0	5.3	0.3	1,556.4	ND
K. marxianus (S280D)	21.4	3.7	0.2	797.3	ND
C. tropicalis [36]	30–36	NR	NR	9–18	ND
N. crassa [35]	34 ± 4	3,600 ± 200	110	1.8 ± 0.5	16 ± 4
C. parapsilosis [18]	31.5	2,835	90	36.5	3.3
P. stipitis [30]	42	1,500	36	9	21
C. tenuis [12]	72	1,300	18	4.8	25.4
C. shehatae [31]	160[a]	NR	NR	20	150

NR not reported, *ND* not detectable

[a] Determined with NADH as coenzyme

anaerobic culture. The redox imbalance was the key reason for the decreased activity because the yeast would accumulate xylitol during anaerobic culture.

The tetra-amino acid motif Ile276-Pro277-Lys278-Ser279 conserved among NADPH-dependent reductases [17, 18] can be found (enclosed in rectangle box in Fig. 2) in the amino acid sequences of the KmXR proteins. Lys-270 (Lys278 in KmXR) in this motif of *Pichia stipitis* XR (PsXR) binds to the 2′-phosphate of NADPH [17]. Based on previous mutational studies performed on *Candida tenuis* XR (CtXR), positions Lys-274, Ser-275, and Asn-276 (Lys-278, Ser-279, and Ser-280 in KmXR; designated by inverted triangles in Fig. 2) emerged as key locations for mutation to increase cofactor specificity for NADH over NADPH (Fig. 2) [17, 18, 24]. The *Talaromyces emersonii* $XR^{K271R+N273D}$ double mutant has a 16-fold increase in the coenzyme preference for NADH, which shows that lysine and asparagine are the major determinants of NADPH recognition [11]. Additionally, in the PsXR and CtXR, mutations at the same positions greatly increase the preference of XR for NADH [32]. *S. cerevisiae* that express the K274R/N276D double mutant CtXR and XDH from *Galactocandida mastotermitis* have 42% increased ethanol yield and 52% decreased xylitol yield [25]. In KmXR, the amino acid residues Lys278-Ser279-Ser280 correspond with the Lys-Ser-Asn of PsXR and CtXR, whereas Asn was substituted with Ser. Ser-280 possibly affected activity and coenzyme preference. Therefore, site-directed mutagenesis was used to check the affects of this site.

Site-directed mutagenesis of the Ser-280 in KmXR

Both S280 N and S280D mutations improved the enzymatic affinity to xylose but greatly reduced their affinity to the coenzyme NADPH (Table 4). The S280 N mutation activity (2.50 U/mg) was almost two times comparing to the wild-type enzyme (1.295 U/mg) and the S280D mutation (0.85 U/mg) was lower than the wild-type. However, the mutants still have no activity with NADH as coenzyme (Table 4). These results indicated that the mutations at this position would reduce the affinity to the NADPH, but they cannot improve the affinity to NADH. The affinity to NADH might be affected by the amino acid residues at other positions.

KmXyl1 disruption inhibits yeast growth on xylose medium

After the *Kmxyl1* gene was cloned and the recombinant enzyme was characterized, we needed to confirm whether the cloned *Kmxyl1* encoded an XR that catalyzes xylose reduction in the xylose metabolic pathway. After screening the tryptophan dropout SD medium with growth determina-

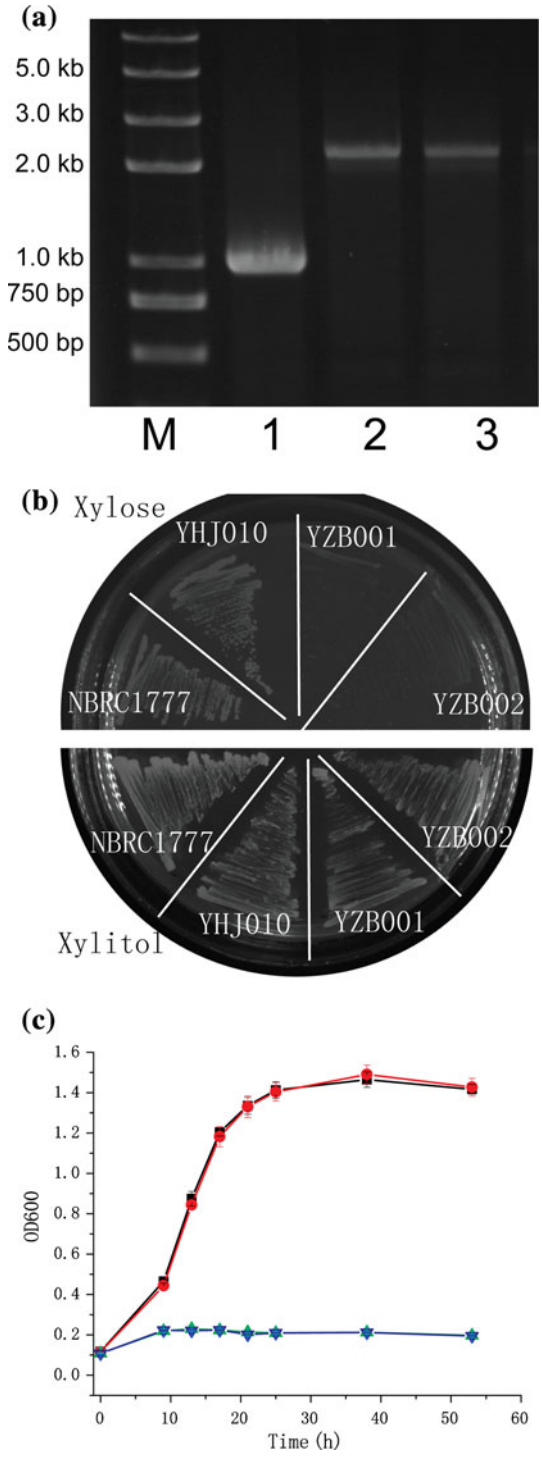

Fig. 4 Disruption of the XR gene *Kmxyl1* inhibits yeast growth on xylose medium. **a** PCR to confirm the disruption of *xyl1*. M: DNA marker, 1: *K. marxianus* YHJ010, 2: YZB001, 3: YZB002. **b** The growth of *K. marxianus* on SD plate with xylose or xylitol as sole carbon source. **c** The growth curve of *K. marxianus* NBRC1777 (*filled square*), YHJ010 (*filled circle*) YZB001 (*filled triangle*) and YZB002 (*filled inverted triangle*) in liquid SD-xylose medium with xylose as sole carbon source

tion the SD medium with xylose (SDX1) or xylitol (SDX2) as sole carbon source, several *Kmxyl1* knockout colonies were obtained. Disruption of the *Kmxyl1* gene in *K. marxianus* was confirmed by PCR (Fig. 4a). Using the genomic DNA from the original strain and disrupted strains as template, bands of about 1 and 2.1 kb were amplified, respectively. The 2.1-kb band consisted of the size shift with the *trp1* gene insertion and proved that the *Kmxyl1* gene in the genome was disrupted (Fig. 4a, lanes 2 and 3). The xylose utilization ability of the *Kmxyl1*-disrupted strain was determined by measuring growth on the xylose medium. On the SDX1 solid medium, the growth of *Kmxyl1*-disrupted strains was poorer than those of NBRC1777 and YHJ010, which have the wild-type *Kmxyl1* (Fig. 4b). However, on the SDX2 solid medium, all the strains grew well (Fig. 4b). In the SDX1 liquid medium, the difference was more evident: *K. marxianus* NBRC 1777 and YHJ010 strains grew well and the OD_{600} reached 1.4 in 25 h, whereas the *Kmxyl1*–disrupted strains grew weakly ($OD_{600} < 0.1$) (Fig. 4c). The result proves that the *Kmxyl1* cloned in the present study is responsible for the decreased xylose reduction of *K. marxianus* NBRC1777.

The disruption of *Kmxyl1* caused a deficiency in xylose utilization, but did not hinder xylitol utilization (Fig. 4b). This suggests that KmXR plays an important role in xylose reduction in the xylose metabolic pathway, and only one *Kmxyl1* gene in the genome is essential for *K. marxianus* growth on xylose.

There was insufficient information on the genetic background of *K. marxianus*, thus, molecular manipulation of the yeast was not performed. The genes, which have the same function as those of other yeast, are often used in *K. marxianus* research [22]. In the present study, *trp1* from *S. cerevisiae* was used to complement the tryptophan auxotroph of *K. marxianus* YHJ010 for transformant selection. The compatibility of the selection marker between *K. marxianus* and *S. cerevisiae* will enhance research on *K. marxianus.*

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