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ORIGINAL PAPER

Identification of a xylulokinase catalyzing xylulose phosphorylation in the xylose metabolic pathway of *Kluyveromyces marxianus* NBRC1777

Rongliang Wang · Ling Zhang · Dongmei Wang · Xiaolian Gao · Jiong Hong

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Abstract Xylulokinase is one of the key enzymes in xylose metabolism and fermentation, and fine-tuned expression of xylulokinase can improve xylose fermentation in yeast. To improve the efficiency of xylose fermentation in *Kluyveromyces marxianus*, the gene *KmXYL3*, which encodes a D-xylulokinase (E.C. 2.7.1.17), was isolated from *K. marxianus* NBRC1777. *KmXYL3* was expressed in *Escherichia coli* BL21 (DE3) cells, and the specific activity of the resulting recombinant purified xylulokinase was 23.5 mU/mg. Disruption of *KmXYL3* resulted in both loss of xylitol utilization and marked decrease in xylose utilization, proving that *KmXYL3* encodes a xylulokinase that catalyzes the reaction from xylulose to xylulose 5-phosphate in the xylose metabolic pathway. The slow assimilation of xylose observed in the *KmXYL3*-disrupted strain indicates that *KmXYL3* is critical for xylose and xylitol utilization; however, *K. marxianus* utilizes a bypass pathway for xylose assimilation, and this pathway does not involve xylitol or xylulose.

R. Wang · D. Wang · X. Gao · J. Hong (✉)
School of Life Science,
University of Science and Technology of China,
Hefei, Anhui 230027, People's Republic of China
e-mail: hjiong@ustc.edu.cn

R. Wang · X. Gao · J. Hong
Hefei National Laboratory for Physical Science at the Microscale,
Hefei, Anhui 230026, People's Republic of China

L. Zhang
School of Life Science and Engineering,
Southwest University of Science and Technology,
Mianyang, Sichuan 621010, People's Republic of China

X. Gao
Department of Biology and Biochemistry,
University of Houston, Houston, TX 77004-5001, USA

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Introduction

Lignocellulose has attracted much attention owing to its sustainability as a source for the production of biofuel and other chemicals. Hemicellulose, which is one of the main components of lignocellulose, can be easily hydrolyzed by acid or enzymes to produce xylose (85–90%) [1]. In addition to glucose, xylose is another major lignocellulose hydrolysis product and is the second most abundant sugar in nature [16]. Xylose fermentation is one of the commonly used processes for converting renewable biomass into energy sources such as ethanol; however, the yield of ethanol production through this process is low.

Many microorganisms are able to utilize xylose through various metabolic pathways. Xylose is converted to xylulose by xylose isomerase (XI) (E.C. 5.3.1.5) in bacteria or by xylose reductase (XR) (E.C. 1.1.1.21) and xylitol dehydrogenase (XDH) (E.C. 1.1.1.9) in eukaryotic microorganisms (Fig. 1). Through the subsequent phosphorylation of xylulose, catalyzed by xylulokinase (XK) (E.C. 2.7.1.17), xylulose enters the phosphopentose pathway, where it finally produces ethanol [11, 31] (Fig. 1). The phosphorylation of xylulose to xylulose 5-phosphate (D-xylulose 5-P) by xylulokinase is a key step in xylose fermentation [32].

The yeast *K. marxianus* is considered as a generally regarded as safe (GRAS) microorganism and has advantages such as short generation time and high growth rate at elevated temperatures (0.86–0.99 h^{-1} at 40°C), with an upper growth limit of 52°C [4, 7]. *K. marxianus* also has great ethanol-producing capacity at elevated temperatures [4, 19, 26] and can thus potentially be applied in simultaneous

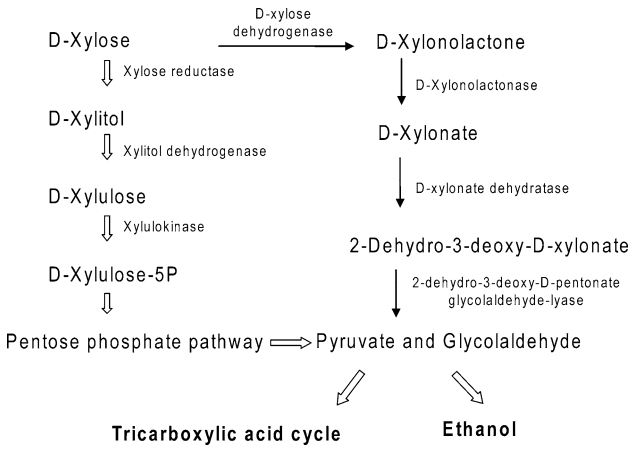

Fig. 1 The normal D-xylose metabolic pathway (*wide arrow*) and the presumed bypass pathway of D-xylose through D-xylonolactone and D-xylonate (*line arrow*) in yeast

saccharification and fermentation (SSF) of lignocellulose and other biomass at temperature higher than 40°C [2, 12], a characteristic that would be useful in the ethanol biofuel industry.

Although *K. marxianus* can assimilate xylose and grow at high temperatures [4], its ability to ferment xylose is poor [4, 5, 24, 33]. Elucidation of the limiting step of xylose fermentation in *K. marxianus* will help to optimize the process of constructing a highly efficient, high-temperature fermentation strain for biomass conversion.

Xylulokinase plays a critical role in xylose metabolism in all natural and engineered microorganisms. The expression level of the enzyme greatly affects xylose fermentation in yeast, and it is reported that fine-tuned overexpression of xylulokinase can improve xylose fermentation to ethanol [14, 22, 32]. Although approximately 30 xylulokinase or potential xylulokinase genes from yeast can be found in GenBank, most of them are hypothetical proteins or theoretically predicted as xylulokinase. Only the xylulokinases from *Saccharomyces cerevisiae*, *Pichia stipitis*, and *Candida maltosa* [13, 21, 27] were cloned, expressed, and characterized. The gene encoding xylulokinase from *Pachysolen tannophilus* was cloned and characterized [30], but the sequence is currently unavailable in GenBank.

In this study, a xylulokinase gene, *KmXYL3*, was cloned from *K. marxianus*. The recombinant enzyme KmXK was expressed in *E. coli* and purified for further experiments. *KmXYL3* gene disruption and KmXK activity characterization confirmed that *KmXYL3* codes for a xylulokinase that catalyzes conversion of xylulose to xylulose 5-P in *K. marxianus*. In addition, the *KmXYL3* disruption experiment indicated the presence of a bypass metabolic pathway that does not involve xylitol and xylulose (Fig. 1). These findings provide important information for future genetic engineering advances to improve xylose fermentation efficiency in *K. marxianus*.

Table 1 Yeast strains used in the study

Strain	Genotype	Phenotype	Source or reference
K. marxianus			
NBRC1777	Wild type	XK wild type	NBRC
YHJ010	*ΔKmura3::Kanr*	XK wild type	[19]
	ΔKmleu2::hisG	URA3, LEU2, TRP1	
	ΔKmtrp1::hisG	Auxotroph	This study
YWRL001	*ΔKmleu2::Kanr*	XK, URA3, LEU2	
	ΔKmleu2::his G	Auxotroph	
	ΔKmtrp1::hisG KmXYL33::Sc TRP 1		

Materials and methods

Chemical and microorganism strains

All chemicals used were of reagent or higher grade and were purchased from Sangon Biotech Co. (Shanghai, China) unless otherwise noted. Yeast extract, peptone, and tryptone were purchased from OXOID (Cambridge, UK). Restriction endonucleases and T4 DNA ligase were from Fermentas China (Shenzhen, China). Xylulose was from Sigma (St. Louis, MO, USA). PrimeStar HS DNA polymerase from TAKARA (Dalian, China) was used for polymerase chain reaction (PCR). *E. coli* DH5 was used as a host cell for all DNA manipulation. *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) containing a protein expression plasmid was used to express the recombinant KmXK. Luria–Bertani (LB; tryptone 10 g, yeast extract 5 g, and NaCl 10 g/l, pH 7.0) medium was used for *E. coli* growth and protein expression with 100 μg/ml ampicillin. *K. marxianus* NBRC1777 (NBRC, Japan) and *K. marxianus* YHJ010 (Δ*Kmura3::Kanr* Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG*, auxotroph strain derived from *K. marxianus* NBRC1777) (Table 1) [19] were grown on solid or liquid YPD medium (peptone 20 g, yeast extract 10 g, and glucose 20 g/l). Synthetic dropout (SD) medium (yeast nitrogen base 6.7 g; glucose, xylose, or xylitol 20 g/l with appropriate amino acids) was used to screen and confirm gene disruption.

Cloning of XK gene *KmXYL3* from *K. marxianus*

The genomic DNA of *K. marxianus* NBRC1777 was extracted as described previously using glass beads and vortexing to break the yeast cells [18]. A partial sequence of *KmXYL3* was amplified by PCR with a pair of degenerated primers (XK-F1 and R1), according to

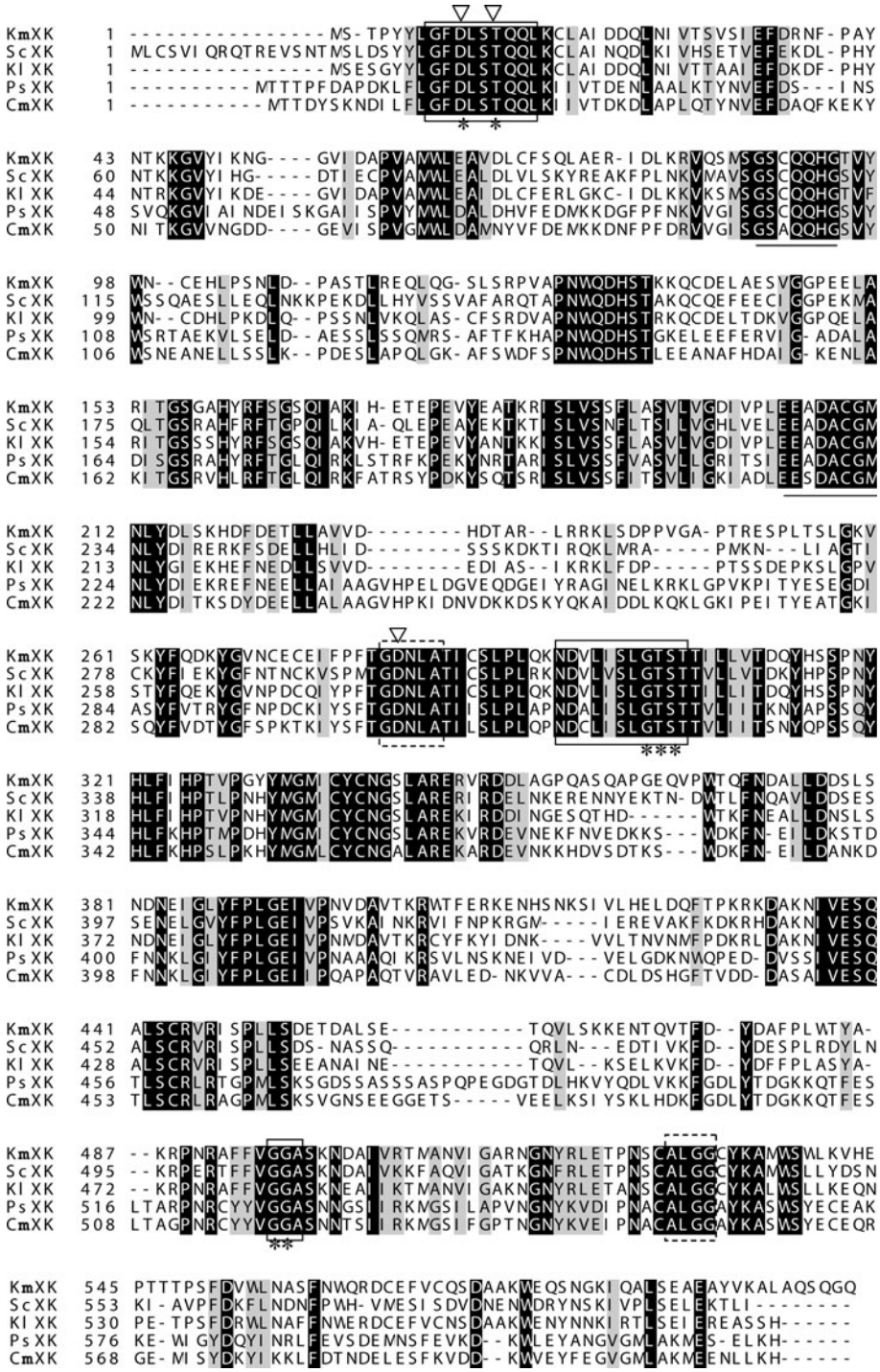

Fig. 2 Sequence alignment of KmXK with other XKs from various microorganisms. The XKs aligned were derived from *K. marxianus* (KmXK, GenBank accession no. GU586191, in this study), *S. cerevisiae* (ScXK, GenBank accession no. EEU06528), *K. lactis* (KlXK, GenBank accession no. XP_454390), *P. stipitis* (PsXK, GenBank accession no. EAZ63302), and *C. maltosa* (CmXK, GenBank accession no. AAY87404). Highly conserved motifs of phosphate 1, phosphate 2, and adenosine are enclosed in boxes. Connect 1 and connect 2 are enclosed in *dashed line boxes*. D10, T13, G302, T303, S304, G496, and G497, indicated by *asterisks*, are amino acids that are common to all sugar kinases. D10, T13, and D282 indicated by *inverted triangles* are catalytic residues and are conserved across the ATPase superfamily

the conserved region GSCQQHG and EEADACGMN (Fig. 2).

After the DNA fragment of the partial *KmXYL3* gene was obtained and sequenced, the unknown flanking sequences were amplified by thermal asymmetric interlaced PCR (TAIL-PCR) [23]. The specific 3′ and 5′ primers for the flanking sequence, which were designed on the basis of the cloned DNA fragment of *KmXYL3*, were XK-FS1, FS2, FS3 and XK-RS1, RS2, RS3 (Table 2). The arbitrary degenerate (AD) primers were AD1, AD2, AD3, and AD4. The arbitrary primer for the second and third round of TAIL-PCR was AC1. The PCR cycle conditions were the same as described by Liu [23]. The amplified flanking sequences were cloned into pMD18-T, and the sequences were validated.

After assembling the flanking sequences with the sequence of the cloned DNA fragment based on the

Table 2 Primers used in this study

Primer name	Sequence (5′→3′)
XK-F1	GGKTCYTGYCARCARCACGG
XK-R1	TTCATWCCACAVGCATCDGC
XK-*Eco*RI-F	ACGTGAATTCAGATGTCTACCCCATACTATTTAGGTTTCG
XK-*Hin*dIII-R	GGGTTCGAAGACTGGCCCTGGCTCTGG
XK-knock-F	TCTACCCCATACTATTTAGGTTTCGATC
XK-knock-R	GACTGGCCCTGGCTCTGGGCCAGG
TRP1-*Eco*RI-F	AGTCGAATTCTTATCCCATTCCATGCGGGGTATC
TRP1-*Eco*RI-R	AGTCGAATTCACTCTTCCTTTTTCAATATTATTG
XK-FS2	CAGTGCGATGAGTTGGCAGAAAGCGTTGGA
XK-FS3	GACCGAGCCCGAGGTTTACG
XK-FS1	GCGAACACTTGCCCTCAAACCTGG
XK-RS1	TCGTAAACCTCGGGCTCGGTCTCGTGGATC
XK-RS2	CCAACGCTTTCTGCCAACTC
XK-RS3	CTTCGCGAACAGTTGCAAGG
AD1	ACGATGGACTCCAGAGAVNVNNNGGAA
AD2	ACGATGGACTCCAGAGABNBNNNGGTT
AD3	ACGATGGACTCCAGAGVVNVNNNCCAA
AD4	ACGATGGACTCCAGAGBBNBNNNCGGT
AC1	ACGATGGACTCCAGAG

* Restriction sites are *underlined*

** 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)

conserved region, the full-length sequence of *KmXYL3* was obtained. The sequence included a complete open reading frame (ORF) and was deposited in GenBank with accession no. GU586191. The DNA sequence of *KmXYL3* was analyzed and aligned with other XK genes using BLAST.

KmXYL3 expression vector construction

The ORF of *KmXYL3* was amplified from the genomic DNA of *K. marxianus* NBRC1777 by PCR using primers XK-*Eco*RI-F and XK-*Hin*dIII-R. The PCR products were then digested with *Eco*RI and *Hin*dIII and inserted into a pET-21c plasmid to construct the expression vector pET-21c-Kmxyl3.

Expression and purification of recombinant KmXK

The pET-21c-Kmxyl3 plasmid was transformed into *E. coli* BL21 (DE3) cells. After obtaining single colonies harboring the pET21c-Kmxyl3 plasmid, one colony was inoculated into 5 ml LB and incubated at 37°C overnight. Thereafter, 4 ml of the preculture was transferred into 400 ml LB medium in a 1-l Erlenmeyer flask and incubated at 37°C. Subsequently, 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration) was added when the OD_{600} was 0.4–0.8, and the incubation was continued for a further 12 h at 16°C. The cells were harvested by centrifugation at 4°C (10 min at 7,000 × *g*) and resuspended in 20 ml buffer A (50 mM Tris–HCl, 25 mM NaCl, pH 8.0). After the cells were lysed by sonication and centrifuged at 4°C (15 min at 15,000 × *g*), the supernatant was loaded onto a Ni–NTA affinity column (Qiagen, The Netherlands). The column was washed with buffer A, containing 5 mM imidazole, and the recombinant KmXK was then eluted using 250 mM imidazole. The recovered KmXK was purified further on a Sephacryl S200 column provided by AKTA UPC10. The purified protein was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

D-Xylulokinase assay

D-Xylulokinase activity was measured as described by Shamanna and Sanderson [28] with some modifications. Xylulokinase activity was assayed in a 50 mM Tris–HCl (pH 7.5) buffered mixture containing glycine (50 mM), KCl (50 mM), ethylenediamine tetraacetic acid (EDTA) (1 mM), NaF (10 mM), phosphoenolpyruvate, tricyclohexylammonium (PEP-Tri) (1 mM), $MgCl_2$ (5 mM), adenosine triphosphate (ATP) (0.5 mM), nicotinamide adenine dinucleotide (NADH) (0.3 mM), lactate dehydrogenase (LDH, E.C. 1.1.1.27, 25 U), pyruvate kinase (E.C. 2.7.1.40, 25 U), prepared xylulokinase

(100 µl), and xylulose (1 mM), and adjusted to a volume of 500 µl with H_2O. The reaction was initiated by adding 1 mM xylulose, and activity was determined by monitoring the decrease of absorbance at 340 nm using a spectrophotometer. NaF inhibited ATPase and NADH oxidase activities but did not affect the activity of D-xylulokinase. One unit of D-xylulokinase was defined as the amount of enzyme that phosphorylates 1 µmol D-xylulose/min. An NADH standard curve was obtained without xylulokinase. The protein concentration of the crude cell extract was measured using the method described by Bradford [8].

Disruption of KmXYL3 in *K. marxianus*

ScTRP1 was amplified from YEGAP [17] by PCR using TRP-*Eco*RI-F and TRP-*Eco*RI-R primers (Table 2) and digested with *Eco*RI. The pET-21c-Kmxyl3 plasmid was digested with *Mfe*I to remove a 338-bp DNA fragment from the *KmXYL3* gene and then ligated with the *Eco*RI-digested *ScTRP1* fragment to construct a new plasmid, pET-21c-Kmxyl3-Trp. The *KmXYL3* disruption cassette was amplified from pET-21c-Kmxyl3-Trp by PCR with primers XK-knock-F and XK-knock-R (Table 2), and transformed by electroporation into *K. marxianus* YHJ010 to disrupt the *KmXYL3* gene. The *KmXYL3* disruptants were selected on SD/-Trp dropout medium, and gene disruption was confirmed by PCR with primers XK-*Eco*RI-F and XK-RS2 (Table 2). The function of *KmXYL3* was validated through carbon utilization on solid or liquid SD medium. *KmXYL3*-disrupted (YWRL001) and wild-type *KmXYL3* strains (NBRC1777 and YHJ010) were inoculated into 5 ml YPD and cultured overnight at 37°C. The cells were then harvested and washed with sterilized water. Finally, the cells were inoculated into 50 ml liquid SD medium with xylose or xylitol as carbon source. The growth of these three strains was measured by monitoring OD_{600} beginning at 0.1.

Results and discussion

Cloning of the *KmXYL3* gene from *K. marxianus*

A 1,968-bp sequence was obtained by assembling the sequence amplified by TAIL-PCR and the sequence amplified on the basis of a conserved region of XKs. The BLAST result showed that it contained a 1,809-bp ORF sequence encoding a 602-amino-acid protein. The putative molecular weight of the protein was estimated to be 68 kDa. The encoded protein was highly homologous to XKs from other microorganisms and belonged to the FGGY family of carbohydrate kinases. The alignment of cloned KmXK and XKs from other microorganisms is shown in Fig. 2. All five sequence motifs [phosphate 1 (residues 10–15), connect 1 (residues 281–287), phosphate 2 (residues 300–304), adenosine (residues 496–498), and connect 2 (residues 529–530)], which are conserved in the ATP-binding regions of the known members of the sugar kinases, actin, and hsp70 class [6], were found in the amino acid sequence of KmXK. In particular, Asp10, Thr13, Gly302, Thr303, Ser304, Gly496, and Gly497 were common to all sugar kinases. Among them, Asp10, Thr13, and Asp282 were catalytic residues and were conserved in the ATPase superfamily (Fig. 2) [10].

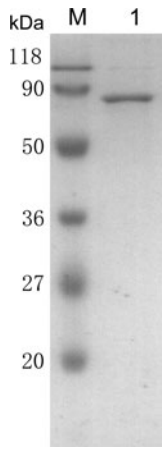

Fig. 3 Purification of xylulokinase. *1* Purified xylulokinase, *M* prestained protein molecular weight marker (Fermentas)

Expression and purification of recombinant KmXK in *E. coli*

Recombinant KmXK was expressed and purified as described in the "Materials and methods." Protein purity was determined by 12% SDS–PAGE analysis (Fig. 3). The molecular weight was estimated to be approximately 68 kDa (71 kDa including amino acids from the vector) as expected. After purification, the specific activity of KmXK was found to be 23.5 mU/mg.

Characterization of recombinant KmXK

KmXK showed enzyme activity with D-xylulose and D-fructose as substrates, and no activity with D-xylose. The specific activity of D-xylulose was 23.5 and 46.0 mU/mg with D-fructose.

Disruption of KmXYL3 in *K. marxianus*

The *KmXYL3*-disrupted strain was obtained as described in the "Materials and methods," and the disruption was confirmed by PCR. As shown in Fig. 4, the amplified product of wild-type *KmXYL3* (*K. marxianus* NBRC1777) was approximately 430 bp long, whereas YWRL001 (the *KmXYL3*-disrupted strain) was approximately 1,300 bp

long, indicating that the *KmXYL3* gene was disrupted by the insertion of *ScTRP1* at the *KmXYL3* position in the genome. When cultured on SD plates with xylose or xylitol as sole carbon source, NBRC1777 and YHJ010, which contained wild-type *KmXYL3*, grew well, whereas YWRL001 was unable to grow on xylitol, and showed poor growth on SD plates with xylose (Fig. 5). Cell growth was also determined in liquid SD medium with xylose or xylitol as carbon source, and the results were similar to those observed on plated medium (Figs. 5, 6). NBRC1777 and YHJ010 grew well in the liquid medium with xylose and xylitol as carbon sources, with OD_{600} reaching approximately 1.2 and 1.4, respectively; however, YWRL001 did not grow on xylitol, and only grew weakly on xylose ($OD_{600} < 0.4$ after a 32 h culture) (Fig. 6). These results show that KmXK encoded by *KmXYL3* catalyzes the xylulose phosphorylation in the xylose metabolic pathway in *K. marxianus* NBRC1777.

The disruption of *KmXYL3* did not completely block the growth on plates containing xylose but did block growth on those containing xylitol. This indicates that there is an additional metabolic pathway, which can utilize xylose without xylulokinase. Jin et al. suggested a pathway from xylulose to arabinitol, which bypasses the step catalyzed by xylulokinase in *P. stipitis* [21]; however, in this study, the *KmXYL3*-disrupted strain was no longer able to utilize xylitol. Our other studies showed that a *K. marxianus* strain with disrupted xylose reductase (XR) gene *KmXYL1* grew poorly in xylose medium, and a strain with the xylitol dehydrogenase (*XDH*) gene *KmXYL2* disrupted was unable to utilize xylitol for growth, but showed weak growth in SD medium containing xylose (data not shown). All these results further proved the presence of a bypass pathway starting from xylose, not xylulose, as reported by Jin et al. By using the MRSD program (metabolic route search and design, http://www.bioinfo.ustc.edu.cn/softwares/mrsd/), a route was found which starts from D-xylose, then to D-xylonolactone, D-xylonate, 2-dehydro-3-deoxy-D-xylonate, and finally to pyruvate (Fig. 1). This route had previously been found in bacteria [25, 29], where the step from D-xylose to D-xylonolactone was catalyzed by D-xylose dehydrogenase (KEGG reaction K14273); D-xylonolactone was converted to D-xylonate by D-xylonolactonase (KEGG reaction K14274); D-xylonate was then converted to 2-dehydro-3-deoxy-D-xylonate by D-xylonate dehydratase (KEGG reaction K14275); and finally, 2-dehydro-3-deoxy-D-xylonate was converted to pyruvate and glycolaldehyde by 2-dehydro-3-deoxy-D-pentonate glycolaldehyde-lyase (KEGG reaction R01782) (Fig. 1) [29]. Although there is no known corresponding enzyme in yeast, several enzymes homologous to bacterial enzymes have been found in yeast. A peroxisomal multifunctional and oxidation protein encoded by the *Fox2* gene in yeast is homologous to D-xylose dehydrogenase [15], and the gluconolactonase (YBR053C) in yeast [9, 20] is homologous to xylonolactonase. Dihydroxy-acid dehydratase gene (Ilv3p, GenBank accession no. EEU04815) in yeast is homologous to D-xylonate dehydratase [3]. Although these enzymes exist in yeast and are not specific for xylose metabolism, it is still possible that xylose was metabolized using these enzymes and resulted in the weak growth of this yeast.

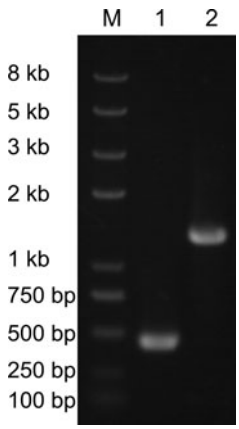

Fig. 4 PCR to identify the disruption of *KmXYL3*. *Lane 1* *K. marxianus* YHJ010, *lane 2*: *K. marxianus* YWRL001 (*KmXYL3* disrupted)

Conclusions

A novel *KmXYL3* gene encoding xylulokinase (KmXK) from *K. marxianus* was cloned. Gene disruption and recombinant enzyme activity both confirmed that KmXK catalyzes xylulose to xylulose 5-P in the xylose metabolic pathway. Disruption of *KmXYL3* hindered assimilation of

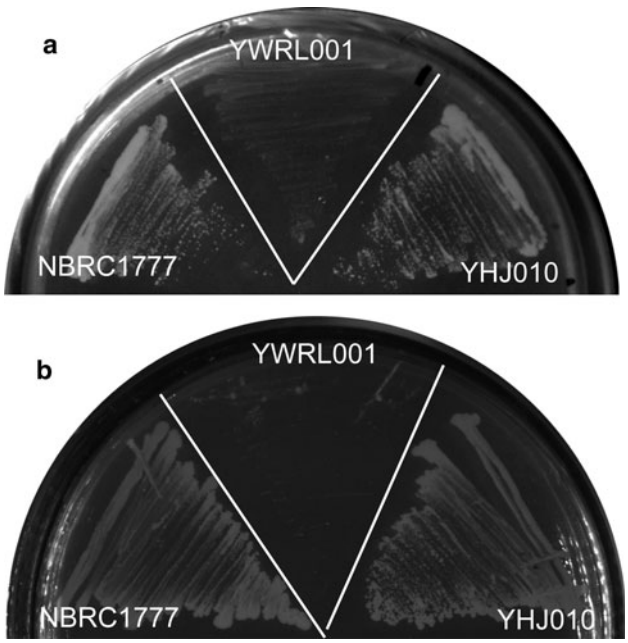

Fig. 5 Growth of *KmXYL3*-disrupted strain *K. marxianus* YWRL001 on SD medium with xylose (**a**) or xylitol (**b**) as sole carbon source

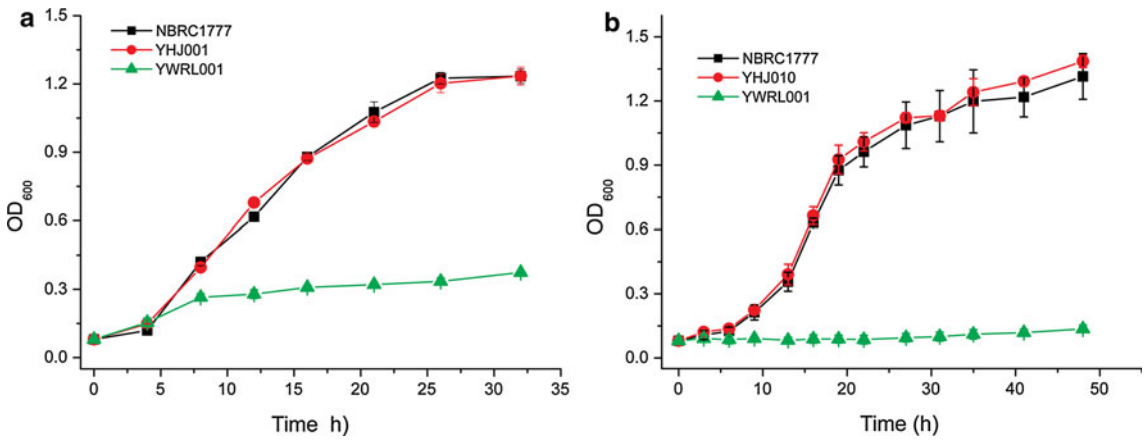

Fig. 6 Growth of *KmXYL3*-disrupted strain YWRL001 in liquid SD medium with xylose (**a**) or xylitol (**b**) as sole carbon source

xylose and xylitol but could not block utilization of xylose completely. This suggests the presence of a bypass pathway starting from xylose. The cloning and disruption of the *KmXYL3* gene provides a foundation for metabolic flux research on xylose and modification of the xylose pathway in *K. marxianus* in the future.

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