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Characterization and evolution of xylose isomerase screened from the bovine rumen metagenome in *Saccharomyces cerevisiae*

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The conversion of abundant levels of xylose in lignocellulosic materials into viable products would generate economic benefits. The heterologous expression of the xylose isomerase (XI) gene is considered a direct and effective strategy for establishing the xylose metabolic pathway in *Saccharomyces cerevisiae*. However, only limited sources of *xylA* are functionally expressed in *S. cerevisiae* and are capable of driving effective xylose consumption. In this study, Ru-*xylA* (where Ru represents the rumen), which was screened from the contents of the bovine rumen metagenomic library, was functionally expressed in *S. cerevisiae*, and the enzyme activity was 1.31 U mg^{-1} protein. This is a new source of XI that can exhibit high activity levels in *S. cerevisiae*. The activity of this enzyme is comparable to those of the *Piromyces* sp. XI. Then, the Ru-XI activity was further improved through mutagenesis and growth-based screening in a centromeric plasmid. A variant containing two mutations (K11T/D220V) that exhibited a 68% increase in enzyme activity was isolated. Our work identified a new xylose isomerase that can be functionally expressed in *S. cerevisiae* and results in a higher XI enzyme activity through mutagenesis.

[**Key words:** Budding yeast; Xylose isomerase; Bovine rumen metagenome; Growth-based screening; Ethanol]

Lignocellulosic biomass is considered one of the most promising feedstocks for sustainable biofuel production. The complete conversion of pentose (~30%) and hexose (~60%) in lignocellulosic feedstocks could generate notable economic benefits in bioethanol production (1). As a traditional ethanol producer, *Saccharomyces cerevisiae* has many positive properties that are beneficial for bioethanol production, such as high ethanol and inhibitor tolerance, high fermentation rates, and high hexose conversion rates. However, *S. cerevisiae* cannot ferment xylose; it can only utilize xylulose, the isomer of xylose (1,2).

Introducing *Scheffersomyces* (*Pichia*) *stipitis* NAD(P)H-dependent xylose reductase (XR) and NAD$^+$-dependent xylitol dehydrogenase (XDH) into *S. cerevisiae* is one of the basic strategies for constructing xylose-utilizing *S. cerevisiae*. However, engineered yeast strains accumulate a significant amount of xylitol because of the cofactor imbalance generated by the different coenzyme specificities of XR and XDH (3,4). Altering the cofactor specificity of XR or XDH or building the conversion pathway between NAD(H) and NADP(H) has decreased but not eliminated xylitol production in recombinant strains (5–9).

Expressing an efficient xylose isomerase (XI) in *S. cerevisiae* is another strategy used to construct the xylose-utilizing *S. cerevisiae* strain (10). XI catalyzes the cofactor-independent reaction that directly converts xylose to xylulose and thus can avoid xylitol production. However, initial work to express xylose isomerase genes was unsuccessful (11), and only the expression of *Thermus thermophilus xylA* in *S. cerevisiae* has shown very low activity (11). Until 2003, *Piromyces* sp. *xylA* (*Pi-xylA*) was determined to be functionally expressed in *S. cerevisiae*, which led to significant xylose metabolism in the engineered strain (10,12). Subsequently, a few XI genes were isolated and functionally expressed in *S. cerevisiae*, including the *xylA*s cloned from *Clostridium phytofermentans* (13), *Orpinomyces* (14,15), *Bacteroides stercoris* (16), *Arabidopsis thaliana* (17), and *Prevotella ruminicula* TC2-24 (18). The search for new XIs that function in *S. cerevisiae* and the engineering of existing XIs have continued to result in higher enzyme activity (19).

Lee et al. (19) reported on the directed evolution of the *Piromyces* sp. XI for improved activity and found a variant (E15D, E114G, E129D, T142S, A177T, and V433I) with a 77% increase in enzymatic activity. Aeling et al. (20) expressed XI from *Ruminococcus flavefaciens* and improved the activity of the enzyme and its affinity for xylose by modifications to the 5'-end and site-directed mutagenesis. These XI mutants showed better xylose utilization capability when expressed in *S. cerevisiae*. These studies highlight the importance of engineering XIs to improve xylose metabolism.

In the present study, new XI genes, which can be functionally expressed in *S. cerevisiae*, were isolated. The enzyme activities of different XIs were compared, and XI from the metagenomic library of bovine rumen contents (Ru-XI) showed slightly higher activity than XI from *Piromyces* sp. in *S. cerevisiae*. Through mutagenesis and growth-based screening, a variant containing two mutations in Ru-*xylA* (K11T/D220V) that exhibited a 68% increase in enzyme activity was obtained.

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MATERIALS AND METHODS

Strains, plasmids, and gene cloning The *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table S1. *Escherichia coli* DH5α was used for subcloning.

The open reading frame (ORF) of *Sc-xylA* (GenBank accession number: 5810100) was cloned from *Sorangium cellulosum* genomic DNA using the primers ScXIF and ScXIR. The amplified fragment was then ligated between the *Bam*HI (isocaudamer enzyme ligation with the *Bgl*II site in the *Sc-xylA* PCR fragment) and *Pst*I sites of plasmid pJFE3 (21) for expression under the control of the *TEF1* promoter and *PGK1* terminator.

The degenerate primers XIcF and XIcR, which were designed based on the two conservative regions of the different XIs (VXW (GP)GREG(YSTA)E and (LIVM) EPKPX(EQ)P) (22), were used to isolate the potential XI gene fragments from four metagenomic libraries, bovine rumen contents, elephant feces, forest soil, and forest kutsuki. The fragments obtained from this step were sequenced and BLAST aligned using NCBI analysis tools (http://blast.ncbi.nlm.nih.gov/). The fragments that were homologous with the *E. coli* XI gene were abandoned. Subsequently, the flanking sequences of these fragments were obtained using the genome-walking method provided in the Genome Walking Kit manual (Takara, Tokyo, Japan). Then, the complete ORF of Ru-*xylA* (GenBank accession number: JF496707) was obtained using the primers RuXIF and RuXIR. The ORF was ligated between the *Bam*HI and *Pst*I sites of the plasmid pJFE3 for expression.

The respiratory-deficient strain BSPX013 (*Pi*-XI, XK, *gre3*:PPP, *cox4Δ*, AE), which contains the well-modified downstream metabolic pathway of xylose and unknown beneficial mutations for xylose metabolism (21), was cultured in YPD medium containing 2% glucose, 2% polypeptone and 1% yeast extract for 24 h. Then, the culture was transferred to the yeast nitrogen base (YNB) medium, which contained 6.7 g l^{-1} of yeast nitrogen base (Sangon Biotech Co., Ltd., Shanghai, China) and 20 g l^{-1} of glucose, was adjusted to a pH of 6.5, and was supplemented with 100 μg/ml of 5-fluoroorotic acid and 50 μg/ml uracil. BSPX042 (XK, *gre3*:PPP, *cox4Δ*, and AE), which has lost the *Pi*-XI plasmid and cannot grow on xylose as the sole carbon source, was selected and assayed for its XI activity and was used as the host strain. The recombinant plasmids containing the different XI expression cassettes were introduced into BSPX042, and the resulting strains were used to evaluate the function of the potential XI genes. The empty plasmid, pJFE3, was also transformed into BSPX042; the resulting strain was named BSGX000 and was used as the reference strain.

Phylogenetic tree and homology analysis The phylogenetic tree was constructed using the Cobalt Constraint-based Multiple Protein Alignment Tool of NCBI (23). The amino acid sequences of the XIs were obtained from the GenBank database. The homology of the XIs was analyzed using the BLAST provided by NCBI. The identities correspond to exact matches and the positives are similarities based on the scoring matrix used.

Xylose isomerase activity assays Overnight-cultured yeast cells were transferred into fresh YNB medium (containing 6.7 g l^{-1} of yeast nitrogen base and 20 g l^{-1} of glucose, pH 6.5) at an initial OD_{600} of 0.5 and then harvested when the OD_{600} reached 4.0. The collected cells were washed twice with sterile water and lysed using a FastPrep cell homogenizer (Thermo Savant, USA) as described previously (21). The homogenized cells were centrifuged at 13,000 rpm for 10 min, and the supernatant was used as a crude enzyme. The protein concentration was measured using a BCA protein assay reagent kit (Sangon Biotech).

The XI activity of the strains was determined at 30°C by measuring the decrease in NADH concentration. The crude enzyme was appropriately diluted to make the reaction last a suitable amount of time. A Helios Gamma Spectrophotometer (Thermo Electron, USA) was employed to measure the absorption values at 340 nm. A water cycling device was connected to the water bath and the spectrophotometer to control the temperature of the reaction cells. The assays were performed in reaction mixtures containing 0.15 mmol l^{-1} of NADH, 10 mmol l^{-1} of $MgCl_2$, 1 U of sorbitol dehydrogenase (Sigma–Aldrich, USA) in 100 mmol l^{-1} of Tris–HCl (pH 7.5), and appropriately diluted crude cell extracts. The reaction was initiated by adding 500 mmol l^{-1} of xylose as described previously (10).

To determine the physicochemical characterization and catalytic properties of XI, the XI activity was assayed via a two-step method. First, the mixture containing 500 mmol l^{-1} of xylose, 100 mmol l^{-1} of buffer, 10 mmol l^{-1} of $MgCl_2$, and 10 μl of the appropriately diluted cell extract was incubated at different temperatures and pH levels for 10 min. To determine the optimum pH of XI, 100 mmol l^{-1} of sodium acetate buffer was used to maintain a pH of 5–7; 100 mmol l^{-1} of Tris–HCl buffer was used to maintain a pH of 7.5, 8, and 9. To determine the optimum temperature of XI, the pH was maintained at pH 7.5. The reaction was stopped by adding 150 μl of 50% trichloroacetic acid, followed by 2 mol l^{-1} of Na_2CO_3 to neutralize the solution. Second, the xylulose produced in the first step was determined at 30°C in 100 mmol l^{-1} of Tris–HCl (pH 7.5), and then 1 U of sorbitol dehydrogenase (Sigma-Aldrich) and 0.15 mM NADH were added. The disappearance rate of NADH was followed at 340 nm, and the amount of xylulose was determined from calibration curves as described previously (24). One unit of isomerase activity was defined as the amount of crude enzyme required to produce 1 μmol of xylulose per min under the assay conditions.

Protein purification The yeast cells were cultured and harvested when the OD_{600} reached 4.0. The collected cells were washed twice with lysis buffer (50 mmol l^{-1} Tris–HCl, a pH of 7.5, 0.5 mmol NaCl, 10 mmol l^{-1} $MgCl_2$, and 1 mmol l^{-1} PMSF) and lysed using a FastPrep cell homogenizer (Thermo Electron). The homogenized cells were centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a Ni-NTA affinity chromatography column that had been charged with binding buffer (50 mM Tris–HCl, 0.5 M NaCl, and a pH of 7.5) in advance. The column was then washed with washing buffer (50 mM Tris–HCl, 0.5 M NaCl, and 250 mM imidazole, pH 7.5). Afterwards, the bound protein was eluted with elution solution (50 mM Tris–HCl, 0.5 M NaCl, and 250 mM imidazole, pH 7.5). The protein was then concentrated using an ultrafiltration tube.

Mutagenesis and growth-based screening For growth-based screening, the centromere plasmid pJFE1 (8) and the yeast BSPX042 were used for gene expression. The error-prone PCR of the *xylA* gene was performed using a low mutagenesis rate (0–4.5 mutations/kb). The randomly mutated xylose isomerase gene library was then ligated with the pJFE1 plasmid. The library was then transformed into recombinant yeast strain BSPX042. The transformants were then cultured in YNB plates with xylose as the sole carbon source for three days. The colonies of a larger size were selected and the xylose isomerase activities of the strains were determined. The strains with improved activities were then selected and the genes were sequenced to determine the mutant.

TABLE 1. Strains and plasmids used in the current study.

Strain/Plasmid	Description	Source
Saccharomyces cerevisiae strain		
CEN.PK 113-5D	*MATa MAL2-8c SUC2 ura3-52*	Peter Kötter
BSPX013	CEN.PK 113-5D derivative; (−194,-1) *XKS1:: loxP-TEF1p, gre3*(-241, +338)*::TPI1p-RKI1-RKI1t-PGK1p-TAL1-TAL1t-FBA1p-TKL1-TKL1t-ADH1p-RPE1-RPE1t-loxP, cox4::loxP,* {pJX5}, daptive evolution/(*Pi*-XI, XK, *gre3*::PPP, *cox4Δ*, AE)	21
BSPX042	BSPX013 derivative; remove the *Pi*-XI by eliminating the plasmid pJX5/(XK, *gre3*::PPP, *cox4Δ*, AE)	This work
BSGX000	BSPX042 derivative; {pJFE3}/({pJFE3}, XK, *gre3*::PPP, *cox4Δ*, AE)	This work
BSGX001	BSPX042 derivative; {pJX7}/(*Ru-xylA*, XK, *gre3*::PPP, *cox4Δ*, AE)	This work
BSGX002	BSPX042 derivative; {pJX8}/(*Ru-xylA* $^{K11T/D220V}$,, XK, *gre3*::PPP, *cox4Δ*, AE)	This work
BSGX003	BSPX042 derivative; {pJX6}/(*Sc-xylA*, XK, *gre3*::PPP, *cox4Δ*, AE)	This work
E. coli strain		
BL21	*F*, *ompT, gal, dcm, lon, hsdS$_B$(r_B m_B)*	TransGen Biotech, China
Plasmid		
pJFE1	YCplac33 derivative; 2μ, *Amp^r*, *URA3 TEF1p-PGK1t*	This work
pJFE3	UEplac195 derivative; 2μ, *Amp^r*, *URA3 TEF1p-PGK1t*	21
pJX5	pJFE3; *TEF1p-Pi-xylA-PGK1t*	21
pJX6	pJFE3; *TEF1p-Sc-xylA-PGK1t*	This work
pJX7	pJFE3; *TEF1p-Ru-xylA-PGK1t*	This work
pJX8	pJFE3; *TEF1p-Ru-xylA$^{K11T/D220V}$-PGK1t*	This work
pJM0	pJFE1; *TEF1p*-Ru-*xylA-PGK1t*	This work
pJM1-6	pJFE1; *TEF1p*-Ru-*xylA mutants-PGK1t*	This work
pET-15b	*Amp^r*, *T7p-T7t*, N-His tag, *lacI*	This work