J Ind Microbiol Biotechnol (2012) 39:1477–1486
DOI 10.1007/s10295-012-1153-6

BIOTECHNOLOGY METHODS

An improved method of xylose utilization by recombinant *Saccharomyces cerevisiae*

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Received: 23 January 2012 / Accepted: 28 May 2012 / Published online: 28 June 2012

Abstract The aim of this study was to develop a method to optimize expression levels of xylose-metabolizing enzymes to improve xylose utilization capacity of *Saccharomyces cerevisiae*. A xylose-utilizing recombinant *S. cerevisiae* strain YY2KL, able to express nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-dependent xylose reductase (XR), nicotinamide adenine dinucleotide (NAD^+)-dependent xylitol dehydrogenase (XDH), and xylulokinase (XK), showed a low ethanol yield and sugar consumption rate. To optimize xylose utilization by YY2KL, a recombinant expression plasmid containing the *XR* gene was transformed and integrated into the *aur1* site of YY2KL. Two recombinant expression plasmids containing an nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent XDH mutant and XK genes were dually transformed and integrated into the 5S ribosomal DNA (rDNA) sites of YY2KL. This procedure allowed systematic construction of an *S. cerevisiae* library with different ratios of genes for xylose-metabolizing enzymes, and well-grown colonies with different xylose fermentation capacities could be further selected in yeast protein extract (YPX) medium (1 % yeast extract, 2 % peptone, and 2 % xylose). We successfully isolated a recombinant strain with a superior xylose fermentation capacity and designated it as strain YY5A. The xylose consumption rate for strain YY5A was estimated to be 2.32 g/gDCW/h (g xylose/g dry cell weight/h), which was 2.34 times higher than that for the parent strain YY2KL (0.99 g/gDCW/h). The ethanol yield was also enhanced 1.83 times by this novel method. Optimal ratio and expression levels of xylose-metabolizing enzymes are important for efficient conversion of xylose to ethanol. This study provides a novel method that allows rapid and effective selection of ratio-optimized xylose-utilizing yeast strains. This method may be applicable to other multienzyme systems in yeast.

Keywords *Saccharomyces cerevisiae* · Xylose utilization · Recombinant yeast · Xylitol · Ethanol

Introduction

Bioethanol is one of the most promising alternatives to petroleum-based fuels. Lignocellulosic biomass, such as wood and agricultural residues, is an attractive feedstock for bioethanol production because of its relatively low cost, abundance, sustainable supply, and minimal conflict with food supply [23, 25]. Lignocellulosic materials are mainly composed of cellulose, hemicellulose, and lignin. Among them, only cellulose and hemicellulose can be used to produce ethanol by fermentation of monomeric sugars obtained using saccharification technologies, including chemical or enzymatic hydrolysis. The cellulose and hemicellulose fractions are composed of glucose and xylose, respectively, which are the most abundant sugars in lignocellulosic biomass [29]. Thus, a cofermenting strain with a capacity for efficient conversion of glucose and xylose to ethanol is indispensable to the lignocellulosic ethanol industry.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-012-1153-6) contains supplementary material, which is available to authorized users.

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Saccharomyces cerevisiae is believed to be the most attractive ethanol-producing microorganism because of its high ethanol productivity, high tolerance to inhibitory compounds, and safety as a generally recognized as safe (GRAS) organism [6, 26]. However, native *S. cerevisiae* strains cannot convert xylose to ethanol [34]. Therefore, to achieve economically feasible ethanol production from lignocellulosic biomass sources, genetically engineered *S. cerevisiae* has been developed to improve this conversion capacity. Numerous efforts have focused on the initial xylose metabolic pathway in *S. cerevisiae* because of its critical role in xylose utilization. In general, initial xylose metabolic pathways include the xylose reductase–xylitol dehydrogenase (XR-XDH) and xylose isomerase (XI) systems [7, 25, 33]. The XR-XDH system comprises XR and XDH, whereas the XI system comprises XI (Fig. 1). Both systems have advantages and challenges for ethanolic xylose fermentation by *S. cerevisiae*. The difference in cofactor preference between the mainly nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-dependent XR and nicotinamide adenine dinucleotide (NAD^+)-dependent XDH in the XR-XDH pathway results in cofactor imbalance and xylitol accumulation during xylose fermentation, which limits the conversion of xylose to ethanol. Xylitol formation in recombinant *S. cerevisiae* could be reduced by expressing mutated XR or XDH with an altered cofactor affinity or by increasing the NADPH pool through overexpression of the heterologous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme [2, 11, 28, 35]. Furthermore, XR and XDH activity levels and the activity ratio of XR-XDH affect xylitol formation during xylose fermentation [17, 24]. Increase in XR and XDH activities, and with a lower XR-XDH activity ratio, was thought to be a way to significantly reduce xylitol accumulation and improve xylose utilization [1, 10, 12]. In the XI system, the introduction of XI instead of expressing XR and XDH to convert xylose into xylulose may allow less byproduct formation and avoid the cofactor imbalance, but expression of the sole XI gene in *S. cerevisiae* did not result in appreciable growth on xylose [15, 18, 19, 21]. Direct comparison of two isogenic strains with high-level expression of the XR-XDH and XI pathways revealed that higher ethanol yield was observed with the XI-carrying strain but that higher xylose consumption rate and specific ethanol productivity were observed with the XR-XDH pathway, indicating that the strain with XR-XDH pathway had a higher rate of xylose fermentation [18]. On the other hand, endogenous xylulokinase (XK) activity in wild-type *S. cerevisiae* is insufficient to support ethanolic fermentation of xylose; therefore recombinant xylose-utilizing *S. cerevisiae* produces ethanol from xylose while additional copies of *XKS1* are expressed [4, 5, 32]. However, unregulated kinase activity may cause a metabolic disorder [31]. It has been experimentally proved that only fine-tuned *XKS1* expression in *S. cerevisiae* improves ethanol production by xylose fermentation [13, 14]. Thus, in addition to redox balance maintenance, the expression level of each enzyme in the xylose metabolic flux is important for efficient xylose utilization. Although previous studies attempted to control the ratio of enzyme activities using promoters of different strengths or high-copy-number expression plasmids combined with chromosomal integration [16, 24], those methods did not effectively optimize the expression levels of enzymes.

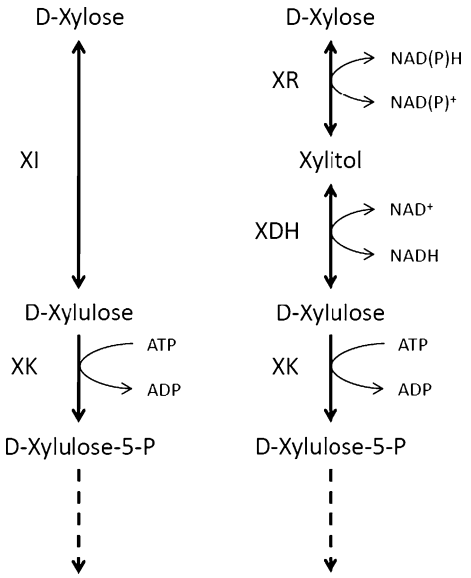

Fig. 1 Initial xylose utilization pathway of xylose reductase–xylose dehydrogenase (XR-XDH) and xylose isomerase (XI) systems

This study aimed at the development of an efficient method to optimize expression levels of enzymes involved in the initial xylose metabolic pathway for improving xylose utilization capacity of recombinant *S. cerevisiae*. Three different chromosomal integration cassettes were constructed to control expression patterns, and recombinant yeast strains with an enhanced capacity to convert xylose to ethanol were obtained. The resulting strains were characterized in a medium containing xylose or a mixture of glucose and xylose.

Materials and methods

Strains and growing conditions

Table 1 shows strains and plasmids used in this study. Yeast strains *S. cerevisiae* and transformants were grown in yeast extract peptone dextrose (YPD) medium (1 % yeast extract, 2 % peptone, and 2 % glucose). For the selection of yeast transformants on YPD or yeast peptone xylose

Table 1 Characteristics of *Saccharomyces cerevisiae* strains and plasmids used in this study

Plasmid and Strain	Relevant genotype	Reference
Plasmid		
pAURlOl	*aur1*	Takara Bio
pB-PGK-CXYLl	*PGKlp-CXYLl-ADH1t*	[3]
pB-PGK-XYL2	*PGKlp-XYL2M-ADHlt*	[3]
pAURCl	*aurl PGKlp-CXYLl-ADHlt*	This work
p5SXYL2	*5S rDNA PGKlp-XYL2M-ADHlt*	This work
p5SXK	*5S rDNA PGKlp-XK-ADHlt*	This work
Strain		
YY2KL	*ADH2::PGKlp-CXYLl-ADHlt, XKSlp::PGKp, HO::PGKIp-XYL2-ADHlt, Migl::TEFp-TAL1-TEFt*	[3]
YYA1	*YY2KL, aur1::pAURCl*	This work
YY5A	*YY2KL, aur1::pAURCl, 5S rDNA::p5SXYL2M, 5S rDNA::p5SXK*	This work

(YPX) (1 % yeast extract, 2 % peptone, and 2 % D-xylose) medium, 400 mg/l Zeocin or 0.5 mg/l of Aureobasidin A (Takara Bio) was added. *S. cerevisiae* was cultivated at 30 °C, 150 rpm. The strain YY2KL and the plasmids pB-PGK-CXYL1, pB-PGK-XYL2M were provided by Dr. Yun-Peng Chao of Feng Chia University, Taiwan [3].

Escherichia coli DH5a [F^- 80d *lacZΔM15 recA1 end1 gyrA96 thi1hsdR17* (m_k^- r_k^-) *supE44 relA1 deoR*Δ(*lacZYA-argF*) U169] was used for subcloning. *E. coli* transformants were selected on Luria-Bertani (LB) medium [1 % tryptone, 0.5 % yeast extract, and 0.5 % sodium chloride (NaCl)] supplemented with 100 mg/l ampicillin or 25 mg/l Zeocin. *E. coli* was cultivated at 37 °C, 150 rpm.

Construction of the integrating plasmids pAURC1, p5SXYL2M, and p5SXK

Recombinant plasmid pAURC1 bearing the *CXYL1* gene from *Candida guilliermondii* downstream of the *PGK* promoter and upstream of the *ADH1* terminator was constructed on the basis of the pAUR101 shuttle vector (Takara Bio, Kyoto, Japan). The sequence of *PGK-CXYL1-ADH1* was amplified from the pB-PGK-CXYL1 plasmid using the primers PGKKpnIfw: 5′-GGTACCGAGGAG CTTGGAAAGATGCC-3′ and ADH1KpnI/SacIrv: 5′-GGT ACCCTGGAGCTCATGCTATACCTGAG-3′. The *Kpn*I/*Sac*I-digested fragment *PGK-CXYL1-ADH1* was cloned into the *Kpn*I/*Sac*I-digested pAUR101 plasmid. The resulting plasmid was designated pAURC1. Recombinant plasmids p5SXYL2 M and p5SXK bearing 5S rDNA from *S. cerevisiae* and coding sequences of XDHm ($NADP^+$-XDH mutant with four mutations, D207A/I208R/F209S/N211R) and XK from *Pichia stipitis* were constructed on the basis of the plasmid pGAPZαA (Invitrogen). The sequence of 5S rDNA was amplified from the genomic DNA of *S. cerevisiae* BCRC 20270 using the primers 5S rDNA-F: 5′-AGATCTGTCCCTCCAAATGTAAAATGG-3′ and 5S rDNA-R: 5′-GGTACCGTAGAAGAGAGG GAAATGGAG-3′. The *Bgl*II/*Kpn*I digested fragment of 5S rDNA was cloned into the *Bgl*II/*Kpn*I-digested plasmid pGAPZαA. The resulting plasmid was designated pGAP5S. The sequence of *PGK-XYL2 M-ADH1* was amplified from the plasmid pB-PGK-XYL2M using the primers PGKNotIfw-F: 5′-GCGGCCGCGAGGAGCTTGGAAAGATGCC -3′ and ADH1BamHIrv-R: 5′-GGATCCCTGGAGCTCAT GCTATACCTGAG-3′. The *Not*I/*Bam*HI-digested *PGK-XYL2 M-ADH1* fragment was cloned into the *Not*I/*Bam*HI-digested plasmid pGAP5S. The resulting plasmid was designated p5SXYL2M. The genomic region of *P. stipitis*, encompassing the entire coding sequence of XK was amplified using the primers XK-F: 5′-CATATGATGACC ACTACCCCATTTG-3′ and XK-R: 5′-CTGCAGTTAGTG TTTCAATTCACTTTCC-3′. *Nde*I/*Pst*I-digested XK-amplified DNA fragments were cloned into the *Nde*I/*Pst*I-digested plasmid p5SXYL2 M between the *PGK* promoter and *ADH1* terminator. The resulting plasmid was designated p5SXK.

Transformation

Yeast transformation of plasmids pAURC1, p5SXK, and p5SXYL2M was performed by electroporation. YY2KL was transformed with pAURC1 to obtain a new transformant named YYA1. YY5A was derived from YYA1, which was simultaneously transformed with p5SXK and p5SXYL2M in a 1:2 molar ratio, and then selected in YPX synthetic medium with Zeocin (Invitrogen). For electroporation, pAUR101 was linearized with *Stu*I, while p5SXK and p5SXYL2M were linearized with *Sph*I. Culture flasks (250 ml) containing 50 ml YPD medium were inoculated with 1 ml of the overnight inoculum culture. It was allowed to grow for 4–5 h at 30 °C with agitation to attain an

absorbance value of 3 U at 600 nm. Cells were harvested in a clinical centrifuge (4,000 rpm), and the pellets obtained were resuspended in lithium acetate buffer [8 ml sterile water, 1 ml lithium acetate, and 1 ml 10× Tris-ethylenediaminetetraacetate (TE) buffer]. The resuspended pellets were then incubated at 30 °C for 45 min. After incubation, the volume was brought to 50 ml with sterile water and washed twice. After two washings, the pellets were resuspended in 0.1 ml 1 M sorbitol to obtain competent cells. These competent cells (40 µl) were mixed with 1 µg linearized plasmid. This mixed sample (45 µl) was subjected to electric shock at 1,500 volts and 25 microfarads using a Bio-Rad Gene Pulser Xcell (Richmond, CA, USA). Following electroporation, the cells were immediately plated on selective YPX medium containing 400 mg/l of Zeocin. Transformants appeared within 3 days at 30 °C. Well-grown colonies with different xylose fermentation capacities were selected.

Molecular biology techniques

Standard cloning techniques were used as described below. Genomic DNA of *P. stipitis* and *S. cerevisiae* was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases and DNA ligase (New England Biolabs) were used according to the manufacturer's specifications. Plasmid isolation from *E. coli* was performed using the Plasmid Mini kit (Qiagen). Polymerase chain reaction (PCR) amplification of the fragments of interest was performed using Ex*Taq* DNA Polymerase (Takara Bio Inc.). PCR was performed using the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). Transformation of *S. cerevisiae* by electroporation was performed as described previously.

Fermentation

The seed cultures were aerobically grown in 50 ml of YPD in 250-ml Erlenmeyer flasks at 30 °C for 16 h. For ethanol production, 10 ml of the seed culture was inoculated into 50 ml of YPX (1 % yeast extract, 2 % peptone, and 2 % D-xylose) or YPD xylose (YPDX) (1 % yeast extract, 2 % peptone, 2 % D-xylose and 2 % glucose) medium in a 250-ml Erlenmeyer flask. An initial dry cell weight (DCW) of approximately 1.0–1.2 g/l was used. All fermentations were performed at 30 °C with agitation at 150 rpm.

Enzyme assay

To measure enzyme activity, recombinant *S. cerevisiae* strains were cultivated in YPD medium for 16 h at 30 °C and harvested by centrifugation at 4,000 rpm for 5 min at 4 °C. Cells were washed thrice with sterile water and resuspended in Tris–hydrochloride (HCl) (pH 7.0) buffer solution. The cells were disrupted using a Mini-BeadBeater (BioSpec Products). Lysates obtained were centrifuged at 13,000 rpm for 10 min at 4 °C, and supernatants were analyzed for XR, XDH, and XK activities. NADPH-dependent XR activity was measured by monitoring NADPH oxidation at 340 nm in the reaction mixture, as described previously. NAD^+/$NADP^+$-dependent XDH activity was measured by monitoring $NAD(P)^+$ reduction at 340 nm in the reaction mixture, as described previously. The XK reaction forming ADP was coupled with pyruvate kinase (PK) and lactate dehydrogenase (LDH) reactions. XK activity was measured by monitoring NADH oxidation at 340 nm in the reaction mixture, as described previously. One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 µmol $NAD(P)^+$ or NAD(P)H per minute. These enzyme activities were determined using a spectrophotometer (U-2900; Hitachi, Tokyo). Protein concentrations in the cell-free extracts were determined in the Bio-Rad protein assay (Bio-Rad Laboratories Inc.) using bovine serum albumin (BSA) as a standard.

Analysis

Concentrations of ethanol, glucose, xylose, xylitol, glycerol, and acetic acid were determined by high-performance liquid chromatography (HPLC; Jasco, Tokyo) equipped with a refractive index detector. The column used for separation was an HPX-87H ion-exclusion column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated using 5 mM sulfuric acid (H_2SO_4) (mobile phase) at a flow rate of 0.6 ml/min. Cell growth was monitored by measuring the absorbance at 600 nm using a spectrophotometer (U-3000; Hitachi). DCW was determined in triplicate using an infrared moisture determination balance (FD-720; Kett Electric Laboratory, Japan). Cell cultures (10 ml) were washed with sterile water, and DCW was then determined using the infrared moisture balance. All data were obtained from the average values of three independent experiments.

Results and discussion

Construction of yeast strains

The *S. cerevisiae* strain YY2KL with a low xylose utilization capacity was used as a parental host for genetic engineering. The genotype of YY2KL contains genes encoding XR from *C. guilliermondii* and XDH, endogenous xylulokinase (XKS1), and transaldolase (TAL) from *P. stipitis*, which are enzymes involved in xylose

metabolism. Except for the TAL gene, which is under the control of the thyrotroph embryonic factor (TEF) promoter; the other genes are controlled by the phosphoglycerate kinase (PGK) promoter. Both TEF and PGK are constitutive promoters. The parental host used for YY2KL was *S. cerevisiae* BCRC 20270 (Table 1).

Several reports have shown that higher XR activity could increase the xylose uptake rate of *S. cerevisiae* during fermentation, but insufficient XDH activity leads to the accumulation of xylitol (a byproduct) [10, 12, 17, 24]. To increase the XR expression level in YY2KL, the strain was integrated with an extra copy of the *CXYL1* gene by transformation with the chromosomal integration plasmid pAURC1 (Table 1). The plasmid pAURC1 derived from pAUR101 (Takara Bio) contained the *CXYL1* gene under the control of the PGK promoter and the *aurc*1 sequence for integration of expression plasmids into the chromosome of the strain. The newly recombinant yeast strain was selected and named YYA1 (Table 1). To verify the xylose utilization capacity of YYA1, the xylose fermentation capacities of YYA1 and the parental strain YY2KL were evaluated in 250-ml flasks containing 50 ml YPX medium. The result indicated that YYA1 had an appreciably higher xylose consumption rate than YY2KL, but it also excreted more xylitol (Table 3; Fig. 3). Thus, the increased XR activity in *S. cerevisiae* could increase the xylose consumption rate, but insufficient XDH may result in xylitol accumulation. To reduce this accumulation and production of ethanol, the strain was integrated with extra copies of XDH and XK genes by transformation with the chromosomal integration plasmids p5SXYL2M and p5SXK. The plasmids p5SXYL2M and p5SXK derived from pGAPZαA (Invitrogen) contained *XYL2M* (coding sequence of XDHm) and *XKS1* genes, respectively, and both these genes were under the control of the *PGK* promoter. Both plasmids also contained the 5S rDNA sequence from *S. cerevisiae* BCRC 20270 for integration of expression plasmids into the chromosome of the strain (Table 1). The yeast *S. cerevisiae* carries 100–200 copies of the rDNA unit randomly repeated on chromosome XII and has been used to obtain multicopy integrants [9, 16, 22, 27]. Because the expression plasmids could be randomly integrated into the sites of the 5S rDNA sequence, the recombinant *S. cerevisiae* library was constructed with different gene copy ratios of xylose-metabolizing enzymes in the XR-XDH-XK metabolic pathway. Because the recombinant strains with an optimum ratio of enzyme activities grow better on YPX medium than other strains, the optimized strains could be further selected by cultivation on YPX medium. Fourteen well-grown colonies with better xylose fermentation capacities were selected in this manner. To verify the xylose utilization capacities of the selected strains, xylose fermentation was further evaluated in 250-ml flasks containing 50 ml YPX medium (Tables 1, 3). A recombinant strain with a superior xylose fermentation capacity was selected from the 14 strains and designated as strain YY5A (Table 1). Another recombinant strain, strain F, was selected from the same pool, with a lower ethanol yield and a higher xylitol yield in comparison with other recombinant strains, and was used to compare with YY5A (Table 1).

Enzyme activity

The specific activities of XR, XDH, and XK in the parental strain YY2KL and recombinant strains YYA1, YY5A, and F were determined (Table 2). The strain YYA1 had a 6.7-fold increase in XR activity in comparison with YY2KL. This result could be attributed to the integration of heterologous XR genes from *C. guilliermondii.* Higher XR activity resulted in a higher xylose consumption rate by YYA1 and also increased the xylitol yield (Table 3; Fig. 3). XR from *C. guilliermondii* specifically uses NADPH, whereas XDH from *P. stipitis* preferentially uses NAD^+. This may result in excess NADH formation and lack of NAD^+, especially under the higher activity of XR than that of XDH. A number of literature reports indicate that insufficient XDH activity and cofactor imbalance are the main reasons for xylitol accumulation. To address this problem, extra copies of XDHm (four-mutation on XDH, with, D207A/I208R/F209S/N211R) genes were integrated into YYA1 to increase the $NADP^+$-preferring XDH activity of the cells. The $NADP^+$-preferring XDH cofactor balanced with the NADPH-dependent XR, and the higher level of XDH activity may reduce xylitol accumulation. Recombinant strains YY5A and F selected from the same pool with a higher ratio of $NADP^+/NAD^+$ XDH and XK activities were determined. With the increased $NADP^+$-preferring XDH and XK activities, the xylose consumption rate and ethanol yield increased in strains YY5A and F. However, strain F, selected from the same pool as YY5A, had a markedly higher xylitol yield than YY5A (Table 3), probably because of the lower XDH and XK activities. The increased $NADP^+$-XDH level helps increase metabolic flux to the central metabolism [7]. On the other hand, the xylitol formation decreased with decreasing XR/XDH ratio. We supposed that the increased $NADP^+$-XDH level could both help to reach the cofactor balance and match the carbon flux. Therefore, it may be concluded that at the higher ratio of $NADP^+$-preferring XDH/XR, XK/XR activities play a critical role in ethanolic fermentation of xylose.

Xylose fermentation

Ethanolic fermentation of xylose by the recombinant strains YY5A, F (pAURC1, p5SXYL2M, p5SXK), YYA1

Table 2 Specific activities of xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) in the cell extracts of recombinant yeast strains

Strain	XR (U/mg)	XDH (U/mg)		XK (U/mg)
		NAD^+	$NADP^+$	
YY2KL	0.233 ± 0.006	1.721 ± 0.031	0.012 ± 0.002	0.013 ± 0.002
YYA1	1.551 ± 0.041	1.435 ± 0.075	0.015 ± 0.002	0.015 ± 0.001
YY5A	1.657 ± 0.063	1.280 ± 0.097	10.963 ± 0.035	3.111 ± 0.006
F	1.445 ± 0.011	1.392 ± 0.042	2.719 ± 0.005	1.545 ± 0.041

Values are averages of the standard deviations of two independent experiments

Table 3 Summary of xylose fermentation after 24 h showing concentrations and yields[a]

Strain	Xylose consumed (g/l)	Xylitol produced (g/l)	Ethanol produced (g/l)	Xylose consumption (%)	Xylitol yield[b] (g/g)	Ethanol yield[c] (g/g)
YY5A	18.7 ± 0.3	1.5 ± 0.1	6.1 ± 0.3	93.7 ± 0.3	0.10 ± 0.01	0.33 ± 0.01
F	17.9 ± 0.3	3.3 ± 0.1	4.6 ± 0.1	89.0 ± 0.6	0.19 ± 0.01	0.26 ± 0.03
YYA1	16.9 ± 0.3	4.5 ± 0.1	3.4 ± 0.1	82.2 ± 0.5	0.27 ± 0.01	0.20 ± 0.01
YY2KL	9.1 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	44.1 ± 0.3	0.11 ± 0.01	0.18 ± 0.01

[a] Values are averages of standard deviations of two independent experiments

[b] Ethanol yield is expressed in grams of produced ethanol per gram, of consumed xylose after 24 h of fermentation

[c] Xylitol yield is expressed in grams of produced xylitol per gram of consumed xylose after 24 h of fermentation

(pAURC1), and parental strain YY2KL was compared using 20 g/l xylose as the sole carbon source (YPX medium). During the fermentation experiments, cell concentrations of all strains reached approximately 8–9 g/l DCW after 48-h fermentation (Fig. 2). However, growth rates of these strains differed significantly during the first 28 h of fermentation [specific growth rate: YY5A (0.067 h^{-1}) > YYA1 (0.052 h^{-1}) > YY2KL (0.050 h^{-1})]. This could be explained by the different xylose utilization capacities. The parental strain YY2KL and strain YYA1 consumed 44.1 % and 82.2 % of the available xylose in 24 h, respectively, whereas the selected recombinant strains YY5A and F with extra copies of XDH and XK genes were able to consume 89–94 % of the xylose (Fig. 3; Table 3). The results indicate that the xylose consumption rate corresponds to enzyme activity levels (Table 2). The xylose consumption rate of YYA1 with extra XR activities was 1.9-fold higher than that of YY2KL. However, the xylitol yield of YYA1 was threefold that of YY2KL, probably because of the higher XR activities, which resulted in insufficient XDH activity. In addition, the 14 selected recombinant strains integrated with the extra pAURC1, p5SXYL2M, and p5SXK yielded different ethanolic fermentation results in YPX medium (data not shown). These strains showed higher xylose consumption rates and lower xylitol yields than YYA1. Results suggest that the ratio and levels of enzyme activities were important to increase the metabolic flux from xylose to ethanol.

Among all strains, YY5A showed the highest xylose consumption rate (0.40 g/gDCW/h) during fermentation, with an ethanol yield of 0.33 $g/g_{consumed\ xylose}$ and a xylitol yield of 0.10 $g/g_{consumed\ xylose}$. This indicates that the xylose consumption rate of strain YY5A increased 2.67 times over that of the parental strain YY2KL (0.15 g/gDCW/h). The ethanol yield was also enhanced 1.83 times by this optimized method. However, YY5A fermenting in a medium containing a higher xylose concentration (40 g/l) showed a xylose consumption rate of 0.41 g/gDCW/h with an ethanol yield of 0.38 $g/g_{consumed\ xylose}$ and a xylitol yield of 0.09 $g/g_{consumed\ xylose}$. Result indicate that a twofold increase in xylose concentration did not influence the specific xylose consumption rate but could increase the volumetric xylose consumption rate from 0.78 g/l/h (YPX with 2 % xylose) to 1.48 g/l/h (YPX with 4 % xylose) (Fig. 4).

Cofermentation of a glucose and xylose mixture

Ethanolic fermentation of a mixture containing glucose and xylose was examined in YPDX medium (22 g/l glucose and 24 g/l xylose as carbon sources) using YY5A, YYA1, and YY2KL. All strains consumed all the glucose within 8 h. The strain YY5A consumed 96.5 % of the xylose at 24 h, and the maximum ethanol concentration achieved was 17.5 g/l, with a yield of 0.36 g of ethanol per gram of total sugars consumed. The strains YYA1 and YY2KL consumed 84.6 % and 53.3 % of the xylose at 24 h, and the

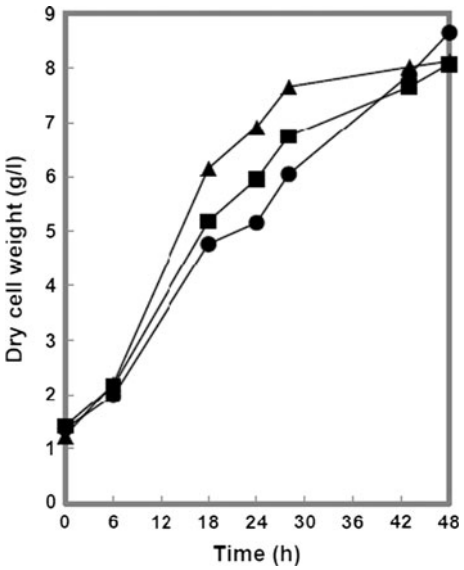

Fig. 2 Aerobic growth of YY2KL (*filled circles*), YYA1 (*filled squares*), and YY5A (*filled triangles*) in yeast protein (YP) medium containing xylose (20 g/l) as the sole carbon source

maximum ethanol concentration achieved was 15.0 g/l and 13.4 g/l, with a yield of 0.31 and 0.33 g of ethanol per gram of total sugars consumed, respectively. The higher ethanol yield of total sugars of YY2KL was contributed mainly from glucose. Xylose consumption by all strains was delayed because of the presence of glucose in the first 4 h. Several studies indicate multiple rate-limiting steps in the metabolic flux from xylose to ethanol for metabolic engineering of *S. cerevisiae* [2, 20]. The transport of xylose in *S. cerevisiae* occurs through nonspecific hexose transporters. The affinity of these transporters for xylose is one or two orders of magnitude lower than that for hexose sugars [8]. This low affinity is considered an early rate-controlling step for ethanolic fermentation of xylose [19, 30].

However, following glucose depletion, the higher volumetric xylose consumption rate could be explained by the increase in cell biomass caused by glucose utilization, but no significant differences were observed in xylitol yields between ethanolic fermentation of xylose only and a glucose/xylose mixture (Fig. 5; Table 4).

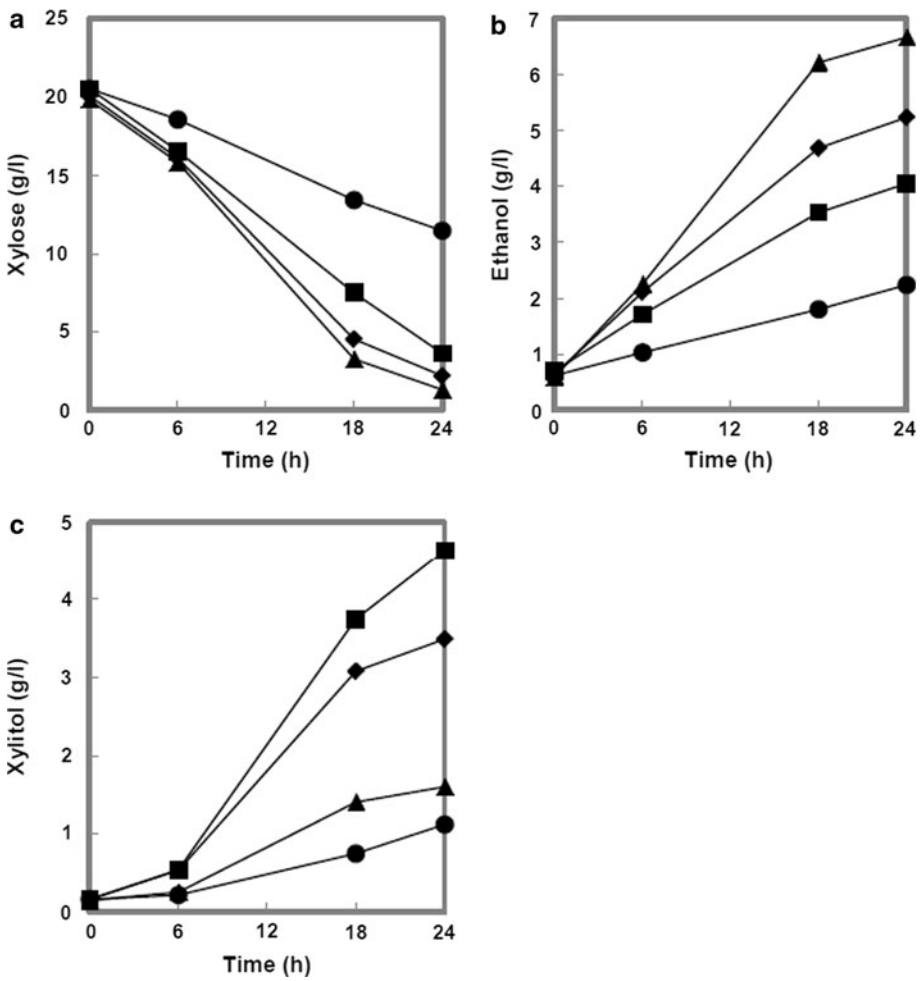

Fig. 3 Comparison of the abilities of recombinant yeast strains YY5A, YYA1, F, and parent strain YY2KL to ferment xylose: **a** xylose consumption; **b** ethanol production; **c** xylitol excretion. *Filled triangles*, YY5A; *filled squares*, YYA1; *filled diamonds*, F; *filled circles*, YY2KL

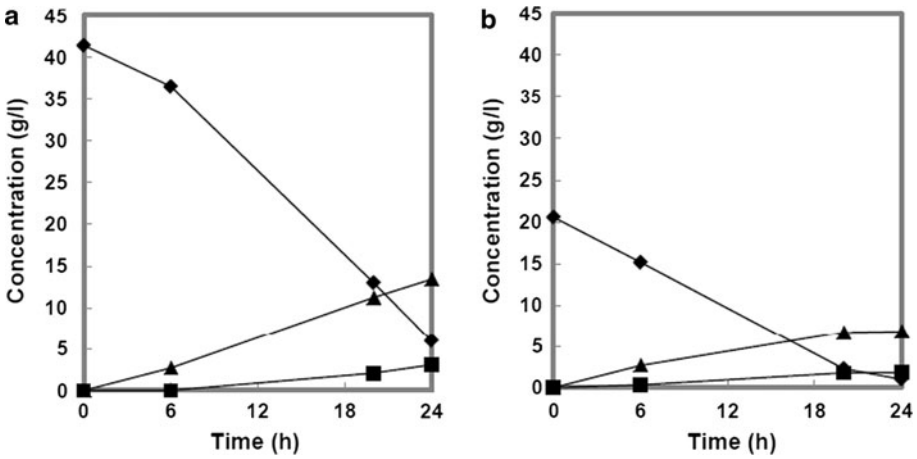

Fig. 4 Comparison of xylose fermentation in yeast protein (YP) with 40 g/l and 20 g/l xylose: **a** YP with 40 g/l xylose; **b** YP with 20 g/l xylose. *Filled diamonds*, xylose; *filled triangles*, ethanol; *filled squares*, xylitol. The same conditions (30 °C, 150 rpm, strain YY5A) were used in all experiments

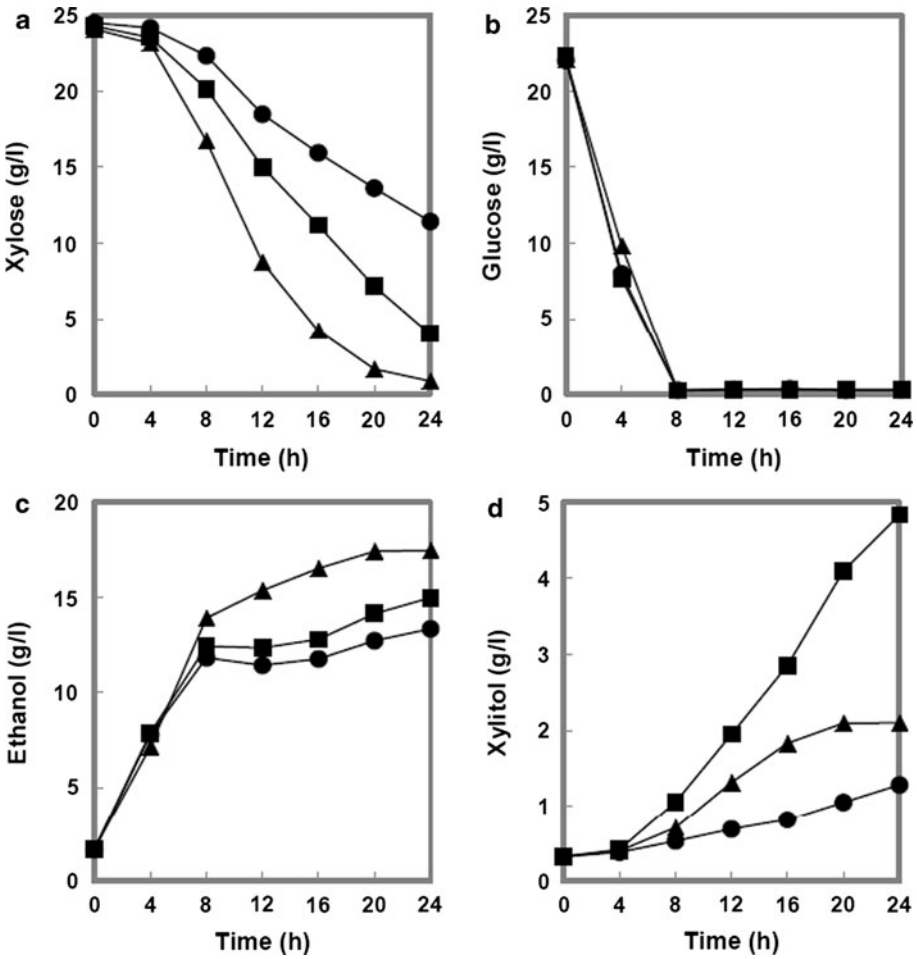

Fig. 5 Comparison of the abilities of recombinant yeast strains YY5A, YYA1, and parent strain YY2KL to coferment glucose and xylose: **a** xylose consumption; **b** glucose consumption; **c** ethanol production. **d** xylitol excretion. *Filled triangles,* YY5A; *filled squares*, YYA1; *filled circles*, YY2KL

Conclusions

An optimal ratio of activities of xylose-metabolizing enzymes is important for the efficient conversion of xylose to ethanol. This study successfully developed a novel method that allows rapid and effective selection of ratio-optimized xylose-utilizing yeast strains. This method may be applicable to other multienzyme systems in yeast. Xylitol accumulation was still observed in the xylose fermentation by strain YY5A. More strains with a different level of enzyme activities will be studied to understand the optimum ratio of enzymes in the pathway and will follow the rule to improve accumulation of xylitol by molecular techniques for further study.

Table 4 Summary of glucose and xylose cofermentation after 24 h showing concentrations and yields[a]

Strain	Xylose consumed (g/l)	Xylitol produced (g/l)	Ethanol produced (g/l)	Xylose consumption (%)	Xylitol yield[b] (g/g)	Ethanol yield[c] (g/g)
YY5A	23.2 ± 0.1	1.8 ± 0.2	15.8 ± 0.2	96.5 ± 0.6	0.08 ± 0.01	0.36 ± 0.01
YYA1	21.7 ± 0.3	4.4 ± 0.3	13.3 ± 0.4	84.6 ± 0.6	0.20 ± 0.01	0.31 ± 0.01
YY2KL	13.1 ± 0.6	1.0 ± 0.1	11.7 ± 0.1	53.3 ± 1.8	0.07 ± 0.01	0.33 ± 0.01

[a] Values are averages of the standard deviations of two or three independent experiments

[b] Ethanol yield is expressed in grams of produced ethanol per gram of consumed xylose after 24 h of fermentation

[c] Xylitol yield is expressed in grams of produced xylitol per gram of consumed xylose after 24 h of fermentation

Glucose was consumed completely in 8 h

Acknowledgments The authors thank Mr. Yi-Feng Jiang and Mr. Mou-Shian Tsay for their technical support with sample analyses.

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