Biotechnol Lett (2015) 37:1623–1630
DOI 10.1007/s10529-015-1840-2

ORIGINAL RESEARCH PAPER

Xylose fermentation by *Saccharomyces cerevisiae* using endogenous xylose-assimilating genes

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Received: 10 March 2015 / Accepted: 11 April 2015 / Published online: 21 May 2015

Abstract
Objectives To genetically engineer *Saccharomyces cerevisiae* for improved ethanol productivity from glucose/xylose mixtures.
Results An endogenous gene cassette composed of aldose reductase (*GRE3*), sorbitol dehydrogenase (*SOR1*) and xylulose kinase (*XKS1*) with a *PGK1* promoter and a terminator was introduced into two *S. cerevisiae* strains, a laboratory strain (CEN.PK2-1C) and an industrial strain (Kyokai No. 7). The engineered Kyokai No. 7 strain (K7-XYL) exhibited a higher sugar consumption rate (1.03 g l^{-1} h^{-1}) and ethanol yield (63.8 %) from a glucose and xylose mixture compared to the engineered CEN.PK2-1C strain. Furthermore, K7-XYL produced a larger amount of ethanol (39.6 g l^{-1}) compared to K7-SsXYL (32 g l^{-1}) with integrated xylose reductase and xylitol dehydrogenase from a xylose-assimilating yeast *Scheffersomyces stipitis* instead of *GRE3* and *SOR1*.
Conclusion The created *S. cerevisiae* strain showed sufficient xylose-fermenting ability to be used for efficient ethanol production from glucose/xylose.

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Keywords Saccharomyces cerevisiae · Xylose · Ethanol

Introduction

Ethanol produced from lignocelullosic biomass is an environmentally friendly alternative to fossil fuels and is attracting interest worldwide. *Saccharomyces cerevisiae* is traditionally used for production of ethanol on an industrial scale. It is attractive because it tolerates high ethanol concentrations and is able to withstand harsh environments. Moreover, various genetic tools for gene modification and fermentation technologies are well established and is regarded as being safe.

Lignocellulosic biomass such as hard woods and straws consists of 20–30 % (w/w) xylose which is the second most abundant sugar. Thus, the development of microorganisms capable of fermenting xylose to ethanol would greatly reduce the overall cost of bioethanol production. As *S. cerevisiae* is not able to ferment xylose, many studies have aimed at metabolically engineering it for xylose utilization and have been focused on introducing genes that encode enzymes of the xylose metabolic pathway by heterologous expression of the xylose reductase/xylitol dehydrogenase (XR-XDH) pathway (Kötter and Ciriacy 1993; Ho et al. 1998; Karhumaa et al. 2007a) or the xylose isomerase (XI) pathway (Walfriedsson et al. 1996; Madhavan et al. 2009). In the XR-XDH pathway, xylose is first reduced to xylitol by xylose reductase (XR), then xylitol is oxidized to

xylulose by xylitol dehydrogenase (XDH). Finally, xylulose is phosphorylated to xylulose 5-phosphate by xylulokinase (XK) and metabolized into ethanol via the pentose phosphate pathway. In the XI pathway, xylose is directly converted to xylulose by bacterial or fungal XI. Although the XI pathway requires no redox cofactors and xylitol production is low, XI-expressing strains exhibit much lower xylose consumption rates than XR-XDH expressing strains (Karhumaa et al. 2007b).

Currently, XR and XDH derived from the xylose-fermenting yeast, *Scheffersomyces stipites*, are widely used as the sources of the gene engineering. *S. cerevisiae* does not naturally utilize xylose as a carbon source although it possesses genes that are homologous to the xylose-assimilating genes. *S. cerevisiae* has six aldo-keto reductases which seem to be involved in conversion of xylose to xylitol. Among them, *GRE3* is thought to be the main xylose reducing enzyme because deletion of the *GRE3* gene was found to decrease xylitol formation by half (Träff et al. 2002). In addition, three genes (*YLR070c*, *SOR1*, *SOR2*) are found in the *S. cerevisiae* genome which are similar to the gene encoding XDH of *Scheffersomyces stipitis* (Richard et al. 1999). Among them, SOR1 enzyme can oxidize xylose to xylitol (Sarthy et al. 1994). We utilized these endogenous genes of *S. cerevisiae* for the purpose of creating a self-cloning host for bioethanol production because the establishment of non-genetically modified yeast would be desirable and suitable for commercial application.

In the present study, we investigated the ethanol fermenting capacities from xylose and a glucose/xylose mixture of a laboratory *S. cerevisiae* strain CEN.PK2-1C, and industrial *S. cerevisiae* strain Kyokai No. 7 engineered with the endogenous xylose-assimilating genes. We also compared the ethanol fermentation performance from a glucose/xylose sugar mixture of Kyokai No. 7 engineered with *S. stipitis* XR (*SsXYL1*) and XDH (*SsXYL2*).

Materials and methods

Strains and media

The plasmids and yeast strains used in this study are listed in Table 1. For pre-cultivation of the yeast cells, YPD medium (20 g peptone l^{-1}, 10 g yeast extract l^{-1}, 20 g glucose l^{-1}) was used. For fermentation, SC medium (0.67 g Difco yeast nitrogen base without amino acids l^{-1} with appropriate amino acids) and CBS medium [7.5 g $(NH_4)_2SO_4$ l^{-1}, 0.75 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 3.5 g KH_2PO_4 l^{-1}, 10.2 g potassium hydrogen phthalate l^{-1}, pH 5.0], each containing an indicated amount of glucose and/or xylose as the carbon source were used. *Escherichia coli* strain JM109 was routinely used for recombinant DNA manipulation. For selection of the transformants, aureobacidin A was added (0.5 mg l^{-1}) to YPD agar plates.

Plasmid and strain construction

Standard techniques of recombinant DNA technology and molecular biology were used in gene cloning and plasmid construction. *GRE3*, *SOR1*, *XKS1* and a *PGK1* promoter and a terminator were amplified from the genomic DNA of CEN.PK.2-1C. *SsXYL1* and *SsXYL2* were amplified from the genomic DNA of *S. stipitis* NBRC 1687. For the enzyme assay, *GRE3*, *SOR1*, *ScXYL2*, *SsXYL1* and *SsXYL2* were amplified by PCR with a *Hin*dIII restriction site and inserted into pGADT7 (Clontech, CA, USA) digested with *Hin*dIII to create pGAD-GRE3, pGAD-SOR1, pGAD-ScXYL2, pGAD-SsXYL1 and pGAD-SsXYL2, respectively. These plasmids were introduced into CEN.PK2-1C to produce CEN-GRE3, CEN-SOR1, CEN-ScXYL2, CEN-SsXYL1 and CEN-SsXYL2, respectively.

Construction of the xylose-assimilating gene expression cassette was performed as follows. The *GRE3*, *SOR1* and *XKS1* genes were each cloned into the *Sal*I site located between the *PGK1* promoter and *PGK1* terminator introduced into pUC18. The *GRE3*, *SOR1* and *XKS1* with the *PGK1* promoter and terminator were each excited with *Eco*RI and *Sph*I. Excited fragments were introduced in tandem into pUC18 by blunt-end ligation to create pUC-XYL. For construction of K7-SsXYL, *XKS1* with a *PGK1* promoter and a terminator was ligated into the same site of pUC18 to yield pUC-XKS1. Next, *SsXYL2* with a *PGK1* promoter and a terminator was blunt-ligated into the *Bam*HI site of pUC-XKS1 to yield pUC-SsXYL2-XKS1. Finally, *SsXYL1* with a *PGK1* promoter and a terminator was blunt-ligated into the *Sma*I site of pUC-SsXYL2-XKS1 to yield pUC-SsXYL. The expression cassettes (*GRE3-SOR1-XKS1* and

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

Strain/plasmid	Description	Source of reference
Plasmids		
pGADT7	2 μ-based multicopy vector, *LEU2*	Clontech
pUC18	Cloning vector	Takara Bio
pAUR135	Cloning vector, *AUR1*	Takara Bio
pGAD-GRE3	pGADT7, intracellular expression of *GRE3*	This study
pGAD-SOR1	pGADT7, intracellular expression of *SOR1*	This study
pGAD-ScXYL2	pGADT7, intracellular expression of *ScXYL2*	This study
pGAD-SsXYL1	pGADT7, intracellular expression of *SsXYL1*	This study
pGAD-SsXYL2	pGADT7, intracellular expression of *SsXYL2*	This study
pAUR-XYL	pAUR135 containing *PGKp-GRE3-PGKt, PGKp-SOR1-PGKt, PGKp-XKS1-PGKt*	This study
pAUR-SsXYL	pAUR135 containing *PGKp-SsXYL1-PGKt, PGKp-SsXYL2-PGKt, PGKp-XKS1-PGKt*	This study
Strains		
CEN.PK2-1C	MATa *ura3-52*, *trp1-289*, *leu2-3 112*, *his3Δ1*, *MAL2-8^C*, *SUC2*	Euroscarf
Kyokai No. 7	Japanese sake yeast strain	National Research Institute of Brewing
CEN-XYL	CEN.PK2-1C, *AUR1*::[*PGKp-GRE3-PGKt, PGKp-SOR1-PGKt, PGKp-XKS1-PGKt*]	This study
K7-XYL	Kyokai No. 7, *AUR1*::[*PGKp-GRE3-PGKt, PGKp-SOR1-PGKt, PGKp-XKS1-PGKt*]	This study
K7-SsXYL	Kyokai No. 7, *AUR1*::[*PGKp-SsXYL1-PGKt, PGKp-SsXYL2-PGKt, PGKp-XKS1-PGKt*]	This study

SsXYL1-SsXYL2-XKS1 with a *PGK1* promoter and a terminator) were digested with *Pvu*II and ligated into pAUR135 which was digested with *Sma*I to generate pAUR-XYL and pAUR-SsXYL, respectively. pAUR-XYL was digested with *Stu*I and transformed into CEN.PK2-1C and K7 to produce CEN-XYL and K7-XYL, respectively. pAUR-SsXYL was digested with *Stu*I and transformed into K7 to produce K7-SsXYL. The yeast was transformed by the lithium acetate method (Ito et al. 1983).

Enzyme activities

Cell extracts for enzyme activity measurement were prepared using Y-PER yeast protein extract reagent (Pierce, Rockford, IL, USA). The activity of XR was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 0.2 mM NADPH with 100 mM xylose as the substrate. The activity of XDH was determined in a reaction mixture containing 50 mM Tris/HCl (pH 8.5) and 1 mM NAD^+ with 100 mM xylitol as the substrate. Protein concentrations in the cell extracts were determined using Quickstart Bradford kit (BioRad) with bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 μmol NAD^+ or NADPH per min.

Fermentation

S. cerevisiae was grown overnight at 30 °C in YPD medium, harvested by centrifugation, washed twice with sterile distilled water to remove traces of glucose, then suspended in an appropriate amount of the water. The cells were inoculated into a 200 ml baffled shake-flask with a filtered silicone plug to avoid ethanol evaporation and containing 50 ml SC or CBS medium with appropriate amino acids. Fermentation was performed at 30 °C with shaking at 140 rpm. The initial dry cell weights were 8.8 g dry cell weight l^{-1} for Kyokai No. 7 and 7.1 g dry cell weight l^{-1} for CEN.PK2-1C.

Analysis of substrates and products

The substrates and products were determined by HPLC using a refractive index detector with a Shodex sugar SP0810 column (300 × 8 mm). The HPLC system was operated at 80 °C using water as the mobile phase at 0.8 ml min^{-1}.

Results and discussion

Enzyme activities of activated endogenous genes

We expressed *GRE3*, *ScXYL2*, *SOR1*, *SsXYL1* and *SsXYL2* on a 2 μ-based multicopy plasmid pGADT7 under the *ADH1* promoter-terminator in CEN.PK2-1C and measured their specific enzyme activities in the cell free extracts of the transformants. The specific enzyme activities are shown in Table 2. The specific activity of *GRE3* was much higher than that of *SsXYL1* in our assay system. *SOR1* showed activities similar to the corresponding enzyme of *S. stipitis*. However, the activity of *ScXYL2* was barely detected, indicating that it does not function in *S. cerevisiae*. This is consistent with the results reported by Toivari et al. (2004) that only low activity was detected with *ScXYL2*. They also reported that *SOR1* showed a xylose-specific response in xylose and glucose culture. Therefore, *GRE3* and *SOR1* were selected as XR gene and XDH gene to create a xylose-assimilating yeast.

Ethanol fermentation from xylose and glucose–xylose mixture

In order to achieve a stable expression of xylose assimilating genes, three genes (*GRE3*, *SOR1*, *XKS1*) arranged in tandem were chromosomally integrated into the *AUR1* locus of CEN.PK2-1C and K7. The obtained recombinant strains (CEN-XYL and K7-XYL) were used for ethanol fermentation from xylose (50 g l^{-1}) in a batch flask culture (Fig. 1; Table 3). CEN-XYL had consumed 45 % of the xylose after 47 h and ethanol reached 7.1 g l^{-1}. Xylitol reached 4.3 g l^{-1}. No glycerol production was observed during the culture. K7-XYL converted xylose to ethanol much faster than CEN-XYL. K7-XYL had completely consumed the xylose in the culture after 47 h and ethanol reached 12.2 g l^{-1}, although the ethanol yield of K7-XYL (46.3 %) per consumed xylose was slightly lower than that of CEN-XYL (51.1 %). K7-XYL produced a large amount of xylitol and glycerol compared to CEN-XYL. Acetic acid production of CEN.PK2-1C gradually increased but remained at a relatively low level (2 g l^{-1}) during fermentation. In contrast, K7-XYL produced a barely detectable level of acetic acid. These results showed that the combination of endogenous xylose-assimilating genes exhibit sufficient activity in xylose fermentation.

Next, a medium containing 80 g glucose l^{-1} and 50 g xylose l^{-1}, representing a typical sugar composition of lignocellulosic biomass, was used to investigate ethanol production by CEN-XYL and K7-XYL (Fig. 2; Table 4). Both strains rapidly fermented glucose followed by xylose. There was no significant difference between the two strains in the rate of glucose consumption. K7-XYL consumed the xylose 1.8 times faster than CEN-XYL, although the amount of the produced ethanol was lower to that in xylose fermentation (2.9 times). When glucose was present in the medium, the rate of consumption of xylose decreased by roughly 30–50 % compared to when xylose was fermented as the only sugar. This may be due to competition between the two sugars for the same sugar transporter during fermentation. A similar observation has been reported with another laboratory strain of *S. cerevisiae* (Gonçalves et al. 2014). Higher rates of xylose consumption and ethanol production have also reported for an industrial strain of *S. cerevisiae* compared to a laboratory strain (Matsushika et al. 2014). The ethanol yield of CEN-XYL per consumed sugar was 73.6 %, which was slightly higher than that of K7-XYL. The ethanol yield of K7-XYL from the initial sugars was much higher (63.8 %) than that of CEN-XYL (58.5 %) indicating the

Table 2 Specific enzyme activities of XR and XDH in cell extracts of the strains

Strain	Specific enzyme activity (U mg^{-1} protein)
CEN-SsXYL1	0.06
CEN-GRE3	0.35
CEN-ScXYL2	0.21
CEN-SsXYL2	2.8
CEN-SOR1	2.1

Cells were cultured in YPD medium. Values are averages of three replicates

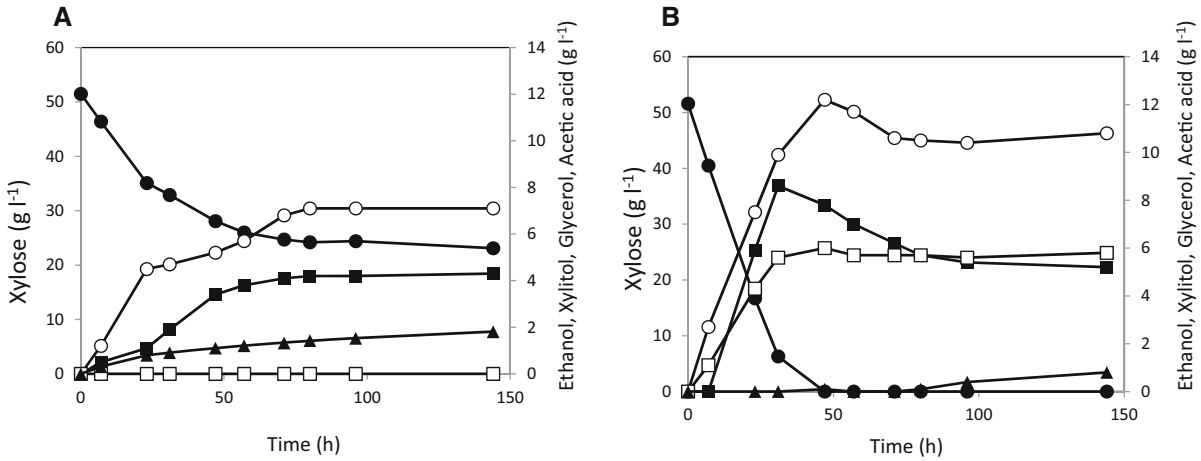

Fig. 1 Fermentation profiles of strains CEN-XYL (**a**) and K7-XYL (**b**) in SC medium containing xylose (50 g l^{-1}) as the carbon source. Xylose, *filled circles*; ethanol, *open circles*; xylitol, *filled squares*; glycerol, *open squares*, acetic acids, *filled triangles*. Data points represent the average of three independent experiments. Deviation was below 10 % of the average

Table 3 Fermentation performance of the recombinant yeasts on xylose culture

Strain	R_x^a	Y_e^b	Y_{xl}^c	Y_{gl}^d
CEN-XYL	0.07 ± 0	0.25 ± 0	0.1 ± 0	0.1 ± 0
K7-XYL	0.20 ± 0.01	0.22 ± 0.01	0.19 ± 0	0.12 ± 0.01

Values are averages of duplicate experiments ± SD

[a] Xylose consumption rate (g xylose^{-1} g-cells^{-1} h^{-1})

[b] Ethanol yield (g ethanol g-consumed xylose^{-1})

[c] Xylitol yield (g xylitol g-consumed xylose^{-1})

[d] Glycerol yield (g glycerol g-consumed xylose^{-1})

superior robustness of the industrial yeast. Urbanczyk et al. (2011) reported that K7 showed high ethanol fermentation activity in spite of low stress tolerance which is consistent with our results showing that K7-XYL has high ethanol productivity compared to CEN-XYL. Therefore, ethanol production should be improved by conferring ethanol tolerance and/or using more ethanol tolerant strains.

Comparison of K7-XYL and K7-SsXYL

SsXYL1 can utilize both NADH and NADPH, whereas *GRE3* uses only NADPH as a cofactor. Therefore, *SsXYL1* is considered to be more advantageous than *GRE3* in that xylitol accumulation is prevented, resulting in a smooth metabolic flow from xylose to ethanol. In reconstitution of xylose assimilation pathway in *S. cerevisiae*, we integrated the endogenous genes responsible for the first three steps of xylose metabolism to construct two types of strains, K7-XYL and K7-SsXYL. K7-XYL possesses xylose-assimilating genes derived from endogenous genes from K7 (*GRE3*, *SOR1* and *XKS*), whereas in K7-SsXYL, *SsXYL1* and *SsXYL2* have been utilized for the first and second steps instead of *GRE3* and *SOR1*, respectively. Since the amount of cofactors which is an essential factor in xylose utilization is closely related to the intracellular redox state, the dissolved O_2 concentration in the culture broth might have an effect on xylitol formation. With the aim of evaluating the effects of aeration on ethanol production, xylose fermentation was carried out with different working volumes of the culture. Figure 3 shows xylose fermentation results for K7-XYL and K7-SsXYL.

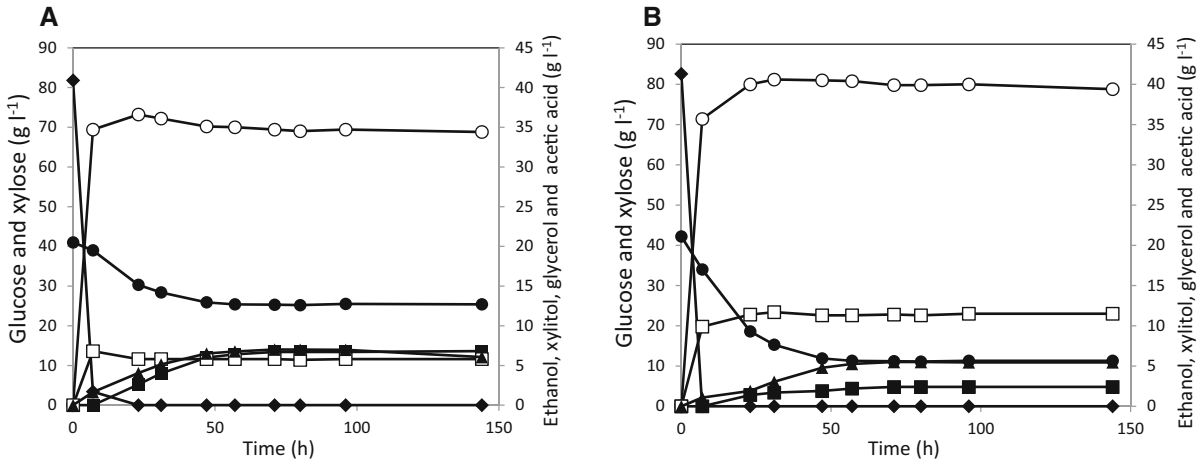

Fig. 2 Fermentation profiles of strains CEN-XYL (**a**) and K7-XYL (**b**) in SC medium containing glucose (80 g l^{-1}) and xylose (50 g l^{-1}) as the carbon sources. Glucose, *filled diamonds*; xylose, *filled circles*; ethanol, *open circles*; xylitol, *filled squares*; glycerol, *open squares*, acetic acids, *filled triangles*. Data points represent the average of three independent experiments. Deviation was below 10 % of the average

Table 4 Fermentation performance of the recombinant yeasts on glucose and xylose culture

Strain	R_g^a	R_x^b	Y_e^c	Y_{xl}^d	Y_{gl}^e
CEN-XYL	1.29 ± 0	0.05 ± 0	0.39 ± 0.01	0.07 ± 0	0.06 ± 0
K7-XYL	1.26 ± 0.02	0.09 ± 0	0.37 ± 0.01	0.02 ± 0	0.11 ± 0

Values are averages of duplicate experiments ± SD

[a] Glucose consumption rate (g glucose^{-1} g-cells^{-1} h^{-1})

[b] Xylose consumption rate (g xylose^{-1} g-cells^{-1} h^{-1})

[c] Ethanol yield (g-ethanol g consumed sugar^{-1})

[d] Xylitol yield (g-xylitol g consumed xylose^{-1})

[e] Glycerol yield (g-glycerol g consumed sugar^{-1})

K7-SsXYL showed almost identical xylose consumption rates regardless of the culture volume (1.82 g l^{-1} h^{-1} with 15 ml and 1.84 g l^{-1} h^{-1} with 30 ml). Increased aeration dramatically decreased xylitol production.

This indicates efficient catalytic activity of *SsXYL2* presumably utilizing NAD^+ supplied by increased O_2 in the culture broth. However, the final ethanol yield from the consumed xylose was improved only slightly by increased aeration (59.1 % with 15 ml and 56.9 % with 30 ml), suggesting the existence of metabolic bottlenecks in the lower pathway from xylitol leading to ethanol production. In the case of K7-SsXYL, increased aeration significantly promoted xylose consumption of K7-XYL (1.47 g l^{-1} h^{-1} with 15 ml and 0.87 g l^{-1} h^{-1} with 30 ml) and ethanol production rate (1.47 g l^{-1} h^{-1} with 15 ml and 0.87 g l^{-1} h^{-1} with 30 ml). Xylitol production was decreased by 56 % in 63 h. These results strongly suggest that the negative effects caused by the cofactor imbalance in the XR-XDH system can be overcome through optimization of the oxygen supply to achieve efficient ethanol production.

We examined ethanol production from a glucose/xylose mixture of the *S. cerevisiae* integrated *SsXYL1*, *SsXYL2* and *XKS1* (K7-SsXYL) in comparison to K7-XYL (Fig. 4). Both strains converted all the glucose within 6 h. However, there was a significant difference in xylose consumption. K7-XYL consumed xylose at a rate of 1.5 g h^{-1} and the xylose was exhausted within 48 h. In contrast, K7-SsXYL converted xylose more slowly than K7-XYL (0.73 g h^{-1}) and consumed only

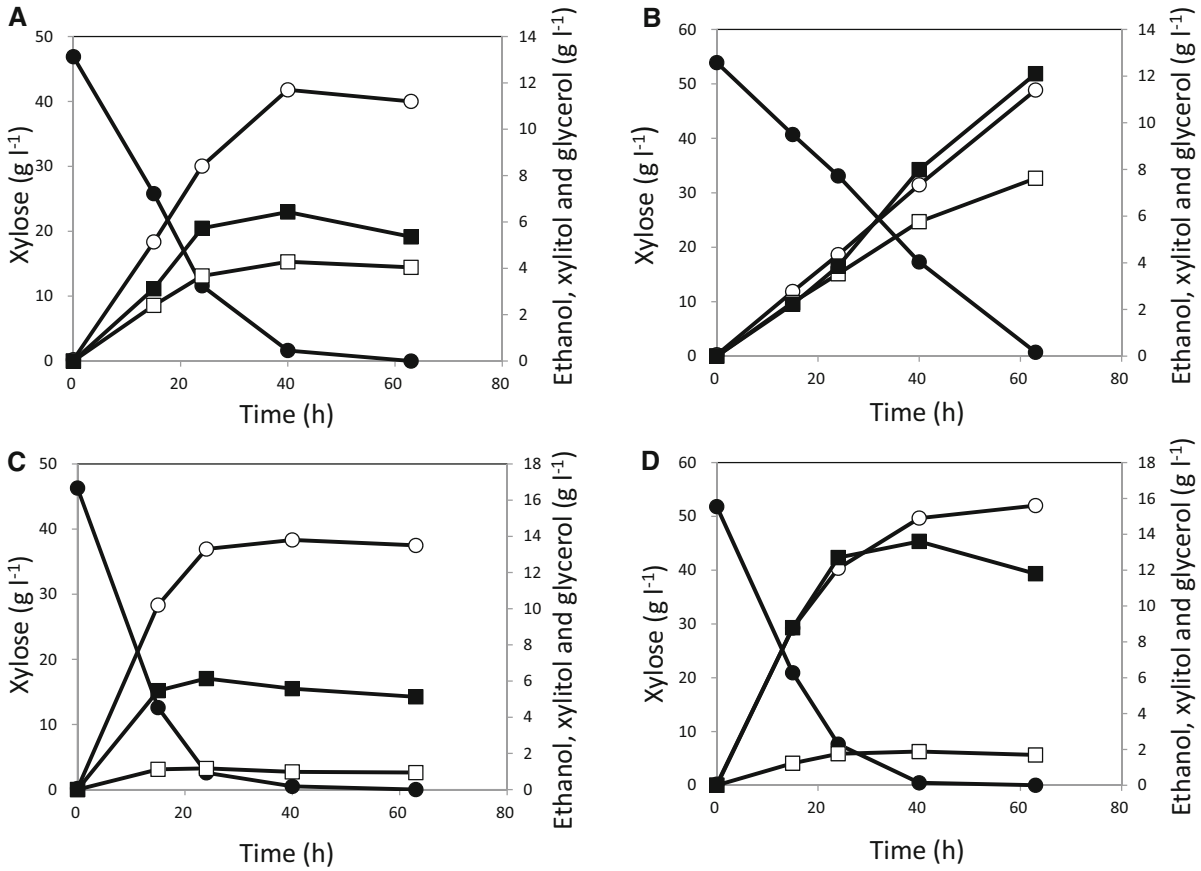

Fig. 3 Fermentation profiles of strains K7-XYL (**a**, **b**) and K7-SsXYL (**c**, **d**) in CBS medium containing xylose (50 g l^{-1}) as the carbon source. Working volume is 15 ml (**a**, **c**) and 30 ml (**b**, **d**). Xylose, *filled circles*; ethanol, *open circles*; xylitol, *filled squares*; glycerol, *open squares*. Data points represent the average of two independent experiments. Deviation was below 10 % of the average

48 % of the xylose even after 72 h of fermentation. As a result, K7-SsXYL produced less ethanol (32 g l^{-1}) compared to K7-XYL (37.6 g l^{-1}). Unexpectedly, K7-SsXYL produced a large amount of acetic acid: 5.31 g l^{-1} after 72 h, which was 40 % higher than that with K7-XYL. This may be one of the main causes of the slow xylose consumption and low ethanol production by K7-SsXYL since 0.5 % v/v acetic acid inhibits xylose assimilation (Limtong et al. 2000). The low ethanol yield from xylose observed with XR-XDH expressing *S. cerevisiae* strains is mainly ascribed to the difference in cofactor preference between XR and XDH, which results in xylitol accumulation. Surprisingly, both strains exhibited low xylitol accumulation despite the difference in the cofactor preferences.

For conversion of xylitol to xylulose by the reaction of XDH, the supply of NAD^+ is assumed to be crucial. The low xylitol production may be attributed to sufficient NAD^+ regeneration presumably generated by adequate oxygen supply mainly from glucose consumption in our culture condition. It is reasonable that xylitol production is affected by degree of NAD^+ regeneration which is closely related to the dissolved O_2 in the culture broth. Therefore, optimization of the culture conditions with a focus on O_2 supply seems to be a promising strategy to further reduce xylitol formation and increase ethanol production. K7-XYL showed a roughly twofold increase in glycerol production compared to K7-SsXYL. The high amounts of glycerol and acetic acid observed with K7-XYL compared to K7-SsXYL seem to be the result of the difference in the intracellular redox state of each strains, although the precise mechanism is not known at present.

In conclusion, the xylose-assimilating *S. cerevisiae* we created showed sufficient xylose fermenting ability to be used for the efficient production of ethanol from a

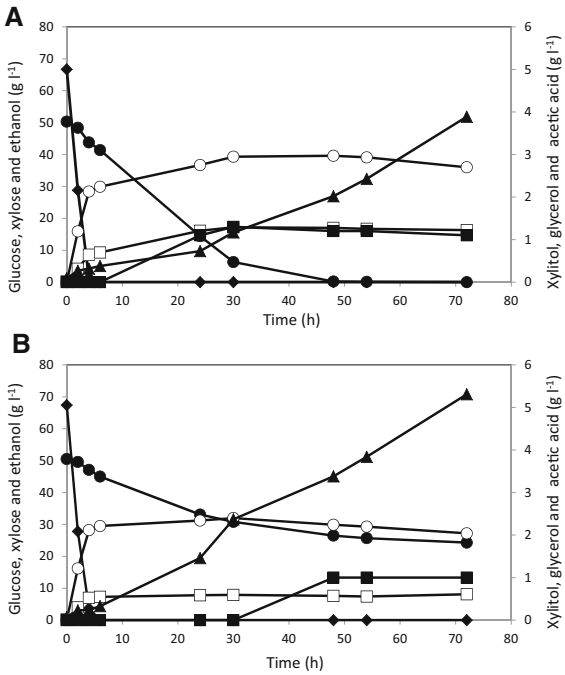

Fig. 4 Fermentation profiles of strains K7-XYL (**a**) and K7-SsXYL (**b**) in CBS medium containing glucose (70 g l^{-1}) and xylose (50 g l^{-1}) as the carbon sources. Glucose, *filled diamonds*; xylose, *filled circles*; ethanol, *open circles*; xylitol, *filled squares*; glycerol, *open squares*, acetic acids, *filled triangles*. Data points represent the average of two independent experiments. Deviation was below 10 % of the average

glucose and xylose mixture. Our results should encourage further studies on the creation of self-cloning strains to be used in industrial ethanol production from lignocellulosic biomass.

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