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Breeding of a xylose-fermenting hybrid strain by mating genetically engineered haploid strains derived from industrial *Saccharomyces cerevisiae*

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Abstract The industrial *Saccharomyces cerevisiae* IR-2 is a promising host strain to genetically engineer xylose-utilizing yeasts for ethanol fermentation from lignocellulosic hydrolysates. Two IR-2-based haploid strains were selected based upon the rate of xylulose fermentation, and hybrids were obtained by mating recombinant haploid strains harboring heterogeneous xylose dehydrogenase (XDH) (wild-type NAD^+-dependent XDH or engineered $NADP^+$-dependent XDH, ARSdR), xylose reductase (XR) and xylulose kinase (XK) genes. ARSdR in the hybrids selected for growth rates on yeast extract-peptone-dextrose (YPD) agar and YP-xylose agar plates typically had a higher activity than NAD^+-dependent XDH. Furthermore, the xylose-fermenting performance of the hybrid strain SE12 with the same level of heterogeneous XDH activity was similar to that of a recombinant strain of IR-2 harboring a single set of genes, XR/ARSdR/XK. These results suggest not only that the recombinant haploid strains retain the appropriate genetic background of IR-2 for ethanol production from xylose but also that ARSdR is preferable for xylose fermentation.

Keywords *Saccharomyces cerevisiae* · Ethanol production · Xylose fermentation · Mating · Xylitol dehydrogenase

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Introduction

Lignocellulosic biomass has been recognized as a potential sustainable source of fermentable sugars and allows for expanding ethanol production without further affecting the food and feed markets [33]. Lignocellulosic hydrolysates, especially those produced from herbaceous and hardwood biomass, are rich in xylose, which is released from hydrolysis of hemicellulose, in addition to glucose from cellulose. Therefore, xylose needs to be fermented to realize the full potential of economic ethanol production from biomass. Although *Saccharomyces cerevisiae* is unable to produce ethanol from xylose, a capacity for fermentation can be conferred by introduction of three xylose-assimilating genes: *XYL1* (xylose reductase, XR) and *XYL2* (xylitol dehydrogenase, XDH) from *Scheffersomyces stipitis*, and *XKS1* (xylulokinase, XK) from *S. cerevisiae* [12].

Xylose fermentation by recombinant *S. cerevisiae* produces xylitol as an undesired byproduct [4]. Moreover, the rate of xylose utilization by recombinant *S. cerevisiae* is generally one to two orders of magnitude lower than that of glucose utilization [10]. Various strategies have been applied to engineer *Saccharomyces* strains capable of efficiently producing ethanol from xylose, including the introduction of xylose transport, improvement of the initial xylose assimilation, changing the intracellular redox balance, and overexpression of pentose phosphate pathways

Table 1 *S. cerevisiae* strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Reference
S. cerevisiae strains		
ENB2004	Haploid of IR-2, *MAT*α	This study
ENB2024	ENB2004, *AUR1::[PGK1p-XKS1-PGK1t, PGK1p-ARSdR-PGK1t, PGK1p-XYL1-PGK1t]*	This study
ENB3002	Haploid of IR-2, *MAT***a**	This study
ENB3009	ENB3002, *AUR1::[PGK1p-XKS1-PGK1t, PGK1p-XYL2-PGK1t, PGK1p-XYL1-PGK1t]*	This study
ENB4003	Mating of ENB2004 and ENB3002, *MAT***a**/α	This study
SE12	Mating of ENB2024 and ENB3009, *MAT***a**/α	This study
MA-R4	IR-2, *AUR1::[PGK1p-XKS1-PGK1t, PGK1p-XYL2-PGK1t, PGK1p-XYL1-PGK1t]*	[28]
MA-R5	IR-2, *AUR1::[PGK1p-XKS1-PGK1t, PGK1p-ARSdR-PGK1t, PGK1p-XYL1-PGK1t]*	[27]
IR-2	Alcohol-fermenting flocculent yeast, *MAT***a**/α	[21]
BY4741	*his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, MAT***a**	[3]
BY4742	*his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, MAT*α	[3]
Plasmids		
pAUR-XKXDHXR	*PGK1p-XKS1-PGK1t, PGK1p-XYL2-PGK1t, PGK1p-XYL1-PGK1t, AUR1-C*	[25]
pAUR-XKARSdRXR	*PGK1p-XKS1-PGK1t, PGK1p-ARSdR-PGK1t, PGK1p-XYL1-PGK1t, AUR1-C*	[25]

[26, 36]. In addition to the higher activity of XDH relative to XR, increased activity of both XR and XDH has been found to be important for generating an efficient xylose-fermenting recombinant *S. cerevisiae* strain [6, 16, 18, 29, 37]. XR is NADPH specific and XDH is NAD^+ specific. To improve the intracellular redox balance, Watanabe et al. constructed a protein-engineered $NADP^+$-dependent XDH (ARSdR) that showed >4,500-fold higher values of k_{cat}/K_m for $NADP^+$ than the wild-type NAD^+-dependent XDH of *S. stipitis* [38]. Recombinant yeast strains with ARSdR show increased ethanol production from xylose accompanied by decreased xylitol production [24, 25, 27, 39].

The selection of host strains is important for practical application of xylose fermentation by recombinant *S. cerevisiae*. Industrial *S. cerevisiae* strains are generally robust and superior ethanol producers under industrial conditions [11]. Some industrial strains have shown more tolerance to lignocellulosic hydrolysates than laboratory strains [9, 23, 34]. For further improvement of industrial strains, mating using haploid strains that have industrially important phenotypes is a useful breeding technology that has provided efficient ethanol production, stress tolerance, and an extended substrate range including xylose to the resultant strains [1, 13, 14, 19, 20, 31, 32, 41].

S. cerevisiae strains have different D-xylulose-fermenting abilities [5, 26, 27, 40], probably because of differences in the pentose-phosphate-pathway flux linking the xylose-to-xylulose pathway to glycolysis [17]. These inherent differences for the xylulose consumption rate have been reported to have an impact on xylose fermentation by recombinant strains [27, 28].

S. cerevisiae IR-2 is an industrial diploid strain having superior xylulose-fermenting performance [27, 28]. The engineered IR-2 strain expressing XR and XDH (or ARSdR) with enhanced XK activity had higher xylose fermentation ability than several other industrial diploid strains [7, 27, 28]; thus, IR-2 is considered to be a promising source of haploids to use for breeding novel xylose-utilizing *S. cerevisiae* strains. The aim of this study was to examine the availability of haploid strains having the genetic background of IR-2 through breeding of diploid strains using the mating reaction. Rapid xylulose-fermenting haploid strains have been successfully isolated from IR-2. Hybrid strains mated with each recombinant haploid strain harboring the three xylose-assimilating genes, XR, XDH (or ARSdR), and XK, were characterized and compared with recombinant xylose-fermenting IR-2 strains.

Materials and methods

Microorganisms and plasmids

The yeast strains used in this study are listed in Table 1. The diploid *S. cerevisiae* IR-2 and its haploid strains were used as the recipient yeast strain for expression of enzymes involved in xylose metabolism. BY4741 (*MAT***a**) and BY4742 (*MAT*α) were used to determine the mating types of haploid strains derived from IR-2. These strains were grown on yeast peptone dextrose (YPD) medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) unless otherwise noted. Aureobasidin A (Takara Bio, Kyoto, Japan) was added at 0.5 mg/L for selection of transformants. Two chromosome-integrating expression plasmids, pAUR-XKXDHXR and pAUR-XKARSdRXR, were constructed

as described previously and used for expression of XR, XDH (or ARSdR), and XK in the recombinant strains [25].

Yeast transformation

The plasmid pAUR-XKXDHXR (or pAUR-XKARSdRXR) digested with *Bsi*WI was transformed into IR-2 and the haploid strains by the lithium acetate method [8], and it was chromosomally integrated into the *aur*1 locus of host strains. Transformation was conducted by the Yeastmaker system (Clontech, Mountainview, CA, USA) following the manufacturer's instructions. Chromosomal integration of the plasmid in the recombinant strain was confirmed by PCR.

Isolation of haploids

IR-2 cells grown on a YPD agar plate were pasted on a sporulation agar plate (10 g/L potassium acetate, 20 g/L agar) and incubated at 30 °C for 5 days to induce sporulation. Haploid strains were isolated by ethanol treatment of the sporulated cells as described by Kurose et al. [22]. A loopful of the sporulated cells was treated with 1 mL of 40 % (v/v) ethanol to kill surviving vegetative cells at 25 °C for 20 min. The spore solution was washed in distilled water and then spread on an eosin–amaranth (EA) agar plate [20 g/L glucose, 1 g/L peptone, 1 g/L yeast extract, 1.5 g/L KH_2PO_4, 1.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L eosin Y (Nacalai Tesque, Kyoto, Japan), 0.02 g/L amaranth (Acid Red 27, Tokyo Chemical Industry, Tokyo, Japan), and 20 g/L agar, pH 5.0] [22]. Haploid and diploid cells were observed as dark red and pink colonies on the EA agar plate, respectively, after incubation at 30 °C for 3 days. The dark red colonies were isolated on a YPD agar or a synthetic defined (SD) agar plate [20 g/L glucose (or 20 g/L xylulose), 0.67 g/L yeast nitrogen base without amino acids (Difco, Detroit, Michigan, USA), 20 g/L agar].

Mating reaction

The haploid culture grown with YPD medium at 30 °C was equally mixed with each BY4741 and BY4742 culture grown under the same conditions. The mixture was incubated overnight at 30 °C and spread on EA agar plates. The mating type was distinguished by the colony color on the plate; mated cells were colored pink, and non-mated cells or haploid cells alone (control) were colored dark red. Hybrid strains were constructed between IR-2-based haploid strains using the same procedure. The mated culture was spread on EA agar plates, and pink colonies were isolated on YPD plates. The isolated strains were stored at −80 °C prior to use.

Enzymatic activities

S. cerevisiae strains were grown with YPD medium for 36 h at 30 °C and harvested by centrifugation at 5,000 ×*g* for 5 min at 4 °C. The protein extract from the yeast cells was prepared following the method of Matsushika et al. [24]. Cells were suspended in Y-PER yeast protein extraction reagent with Halt protease inhibitor cocktail (Pierce, Rockford, IL, USA). The protein concentration was determined by a micro-BCA protein assay kit (Pierce). XR activity in the protein extracts was determined by the change in OD_{340nm} with 200 mM xylose and 0.15 mM NADPH. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of NADPH per min. XDH and ARSdR activity was determined by the change in OD_{340nm} with 333 mM xylitol and 1 mM NAD^+ or $NADP^+$, respectively. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol of NAD^+ or $NADP^+$ per min.

Ethanol fermentation from xylulose

D-Xylulose was prepared by isomerization of D-xylose [30]. Xylose was not detected in the purified xylulose on high-performance liquid chromatography (HPLC) analysis. The haploid and diploid strains were aerobically grown with YPD medium for 24 h at 30 °C. Then, pre-cultures or washed cells were inoculated into 5 mL of YPDXU medium (50 g/L glucose, 20 g/L xylulose, 20 g/L peptone, 10 g/L yeast extract, pH 5.5) at a final concentration of 10 % (v/v) or 0.3 g dry cell/L, respectively. Ethanol fermentation was performed at 30 °C for 72 h in a closed bottle (13 mL) with agitation at 100 rpm. Samples (0.1 mL) of the fermentation broth were removed at appropriate intervals using a syringe and diluted up to tenfold with sterile water. These diluted samples were stored at −30 °C prior to use for HPLC analyses.

Ethanol fermentation from xylose

Two types of media were used for xylose fermentation: YPX medium (10 g/L yeast extract, 20 g/L peptone, and 21 g/L xylose) and YPDX medium (10 g/L yeast extract, 20 g/L peptone, 45 g/L glucose, and 45 g/L xylose). Yeast pre-culture was conducted with 30 mL of YPD medium. Cells were collected by centrifugation and washed with sterile water. After washing, the cells were inoculated at OD_{600nm} of 1.5 into 20 mL of fermentation media in 50 mL serum vials with a butyl stopper and aluminum cap. All fermentations were performed at 30 °C with agitation at 100 rpm. Samples (0.2 mL) of the fermentation broth were taken at the specified intervals and diluted up to fourfold with 8 mM H_2SO_4. These diluted samples were

Table 2 Fermentation performance of haploid and diploid strains in YPDXU medium

Strain	Xylulose consumption rate (g xylulose/L/h)	Ethanol yield[a] (g/g of total consumed sugar)
ENB2004	0.50 ± 0.06	0.41 ± 0.009
ENB3002	0.81 ± 0.03	0.42 ± 0.002
ENB4003	0.73 ± 0.03	0.43 ± 0.002
IR-2	0.64 ± 0.04	0.43 ± 0.007

Values are the average values of the results from two independent experiments. The initial cell concentration for fermentation was adjusted to approximately 0.3 g dry cell/L

[a] Ethanol yield was calculated from the amount of ethanol produced after 72 h of fermentation

stored at −30 °C prior to use for HPLC analyses. Ethanol yield was calculated based on the amount of ethanol (g) produced from the total consumed sugars (g) after 72 h of fermentation.

Analytical methods

Concentrations of glucose, xylose, xylulose, xylitol, ethanol, glycerol, and acetic acid in fermentation broths were determined with HPLC (Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031Plus, Jasco) using an Aminex HPX-87H and Cation H refill guard column (Bio-Rad, Hercules, CA, USA). HPLC was operated at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and with an injection volume of 20 μL.

Results and discussion

Isolation of xylulose-assimilating haploids

To select host strains suitable for breeding by mating, haploid strains having the IR-2 genetic background were isolated. IR-2 formed not only four-spored asci but also many two- and three-spored asci on a sporulation agar plate; thus, haploid strains were randomly isolated by 40 % (v/v) ethanol treatment of sporulated cells. This treatment was developed to isolate ethanol-tolerant haploids [22]. The mating type of the isolated haploid strains was determined by the mating reaction with BY4741 (*MAT***a**) and BY4742 (*MAT*α), and finally 10 and 12 haploid isolates of *MAT***a** and *MAT*α, respectively, were obtained in the present study.

Xylulose-assimilating ability was evaluated for the haploid isolates because this ability is a major characteristic of IR-2 related to efficient xylose fermentation. Ethanol fermentation from xylulose was performed using YPDXU medium containing 50 g/L glucose and 20 g/L xylulose. The pre-culture was inoculated at a final concentration of 10 % (v/v). The xylulose consumption rates of haploid isolates were in the wide range of 0.15–0.55 g/L/h (data not shown). The consumption rates of the control strains BY4741 and BY4742 were both 0.13 g/L/h. These results suggest that the high xylulose-assimilating ability of IR-2 is predicted to be controlled by multiple heterogenic factors. We selected ENB2004 (*MAT*α) and ENB3002 (*MAT***a**) on the basis of maximum xylulose consumption rates. These strains showed no autotrophy when growing on SD agar plates containing glucose or xylulose as a sole carbon source. In addition, the diploid strain ENB4003 (*MAT***a**/α) obtained by the mating reaction between ENB2004 and ENB3002 was confirmed to form spores. The xylulose consumption rates of ENB3002 and ENB4003 in YPDXU medium were significantly faster than that of the parent strain IR-2 when the initial cell concentration for fermentation was adjusted to approximately 0.3 g dry cell/L (Table 2). The diploid strains ENB4003 and IR-2 showed slightly higher ethanol yields at 72 h than the haploid strains; their ethanol yields were both 0.43 g/g of total consumed sugar (Table 2). These results indicate that the haploid strains ENB2004 and ENB3002 can be used as host strains for breeding to incorporate the superior xylulose-assimilating and ethanol-producing capabilities of IR-2.

Mating of xylose-fermenting recombinant haploids

Recombinant haploid strains from ENB3002 and ENB2004, termed ENB3009 and ENB2024, respectively, were constructed for the breeding of xylose-fermenting hybrid strains. To evaluate natural selection during the mating reaction, heterogeneous XDH genes, i.e., wild-type NAD^+-dependent XDH and $NADP^+$-dependent ARSdR, were integrated into the ENB3009 and ENB2024 strains, respectively (Table 1). Thirty hybrid strains obtained by mating the ENB3009 and ENB2024 strains, harboring two sets of xylose-assimilating genes (XRs/heterogeneous XDHs/XKs), were screened based upon growth rates on YPD and YPX agar plates, and six strains (SE4, 5, 6, 12, 13, and 14) were selected. The enzymatic activity for the initial xylose assimilation in the six strains was compared with that of MA-R4 and MA-R5 prepared from recombinant IR-2 with a single set of chromosomally integrated xylose-assimilating genes, XR/XDH/XK and XR/ARSdR/XK, respectively (Fig. 1). The XR activities of six strains were similar to or slightly lower than those of strains MA-R4 and MA-R5, indicating that homogeneous XR genes in the mating strains did not necessarily increase XR activity. Interestingly, significantly higher ARSdR activity than XDH activity was observed in all hybrid strains except for SE12 (Fig. 1), which implies that the redox balance improved by the combination of ARSdR

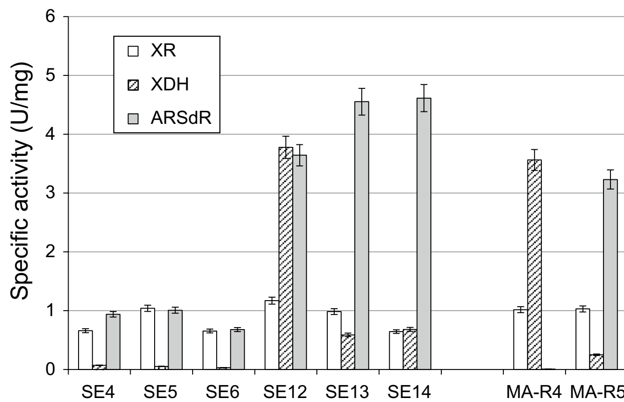

Fig. 1 Activities of xylose reductase (XR), wild-type NAD^+-dependent xylose dehydrogenase (XDH), and engineered $NADP^+$-dependent XDH (ARSdR) in the cell extracts of recombinant yeast strains. Values are the average values from three independent experiments

and XR is preferred by hybrid strains harboring heterogeneous XDHs.

The ARSdR activity of SE4, SE5, and SE6 was significantly lower than that of MA-R5; the XR/ARSdR ratios (ratio of specific enzyme activities) of these strains were 0.7–0.96. In general, a higher level of XDH activity relative to XR activity is preferred for ethanol fermentation from xylose by recombinant yeast [26]. Xylitol formation decreased with a XR/XDH ratio of 0.06–0.1, whereas ethanol formation increased [6, 37]. In contrast, a recombinant diploid strain expressing engineered XR with higher NADH specificity previously showed a low amount of xylitol accumulation and improved ethanol yield at a XR/XDH ratio of 0.7 [20]. The difference in cofactor specificity between XR and XDH seems to require a high level of XDH. These reports suggest that the relatively high ratios of XR/ARSdR in SE4, SE5, and SE6 are closely related to growth selection on YPD and YPX agar plates.

Evaluation of ethanol fermentation from xylose by xylose-fermenting diploid strains

The impact of XDH and ARSdR activities in the xylose-fermenting performance of diploid strains was evaluated in YPX medium containing xylose as the sole carbon source. Strain SE12 possessing equal activities for ARSdR and XDH (Fig. 1), MA-R4, and MA-R5 were used for this analysis. The activity of XR and XDH in strain SE12 was similar to that in MA-R4, and the activity of XR and ARSdR in SE12 was almost the same as that in MA-R5. We found that SE12 exhibited the same fermentation property as MA-R5 except for acetic acid production (Fig. 2). This suggests that ARSdR, but not XDH, was preferentially utilized in the xylose assimilation of SE12 and that the haploid hosts used for the construction of the hybrid strain retained the appropriate genetic background for xylose fermentation. Xylose was almost completely consumed by all strains at 72 h (Fig. 2a). SE12 and MA-R5 showed decreased xylitol release and increased ethanol production relative to MA-R4 (Fig. 2b, c). A similar trend was observed in previous reports using other xylose-utilizing yeast strains with chromosomal integration of pAUR-XKXDHXR or pAUR-XKARSdRXR [24, 25]. SE12, MA-R5, and MA-R4 excreted 0.93, 1.33, 6.65 g/L xylitol at 72 h, corresponding to 4.7, 6.7, and 33 % of the consumed xylose, respectively (Fig. 2b). SE12, MA-R5, and MA-R4 produced 5.80, 5.65, and 4.82 g/L ethanol at 72 h (Fig. 2c); the ethanol yields were estimated at 0.29, 0.28, and 0.24 g per g of consumed sugar, respectively.

SE12 and MA-R5 produced higher amounts of glycerol than MA-R4 (Fig. 2d). Glycerol is not only protective against osmotic stress but also a more favorable by-product than xylitol because less carbon is lost in the three-carbon glycerol than in five-carbon xylitol [2, 18]. Anaerobic glycerol production generally originates from excess NADH in *S. cerevisiae* [15, 35]. However, glycerol production in MA-R4 was not increased after 12 h regardless of accumulation of xylitol caused by excess NADH (Fig. 2b, d).

Evaluation of ethanol fermentation from a mixture of xylose and glucose by xylose-fermenting diploid strains

To evaluate xylose fermentation performance under the presence of xylose and glucose, ethanol fermentation by SE12, MA-R5, and MA-R4 was performed using YPDX medium. The fermentation property of SE12 was very similar to that of MA-R5 (Fig. 3), in accordance with the results observed in YPX medium. There was no difference in glucose consumption among the three strains; glucose was initially consumed within 9 h (Fig. 3a). The xylose consumption rate of SE12 and MA-R5 was significantly increased in the presence of glucose relative to that in YPX medium (Figs. 2a, 3b). At 72 h, SE12 and MA-R5 consumed xylose completely, whereas in MA-R4, 13 g/L xylose remained in YPDX (Fig. 3b). SE12 showed lower production of xylitol than MA-R5 (Fig. 3c), probably because of the increased heterogeneous XDH activity relative to MA-R5 (Fig. 1). The xylitol excreted by SE12, MA-R5, and MA-R4 at 72 h corresponded to 10.9, 16.7, and 50 % of the consumed xylose, respectively. These differences were reflected in ethanol production at 72 h; SE12, MA-R5, and MA-R4 produced 23.3, 22.8, and 18.1 g/L ethanol (Fig. 3d). The ethanol yields of SE12, MA-R5, and MA-R4 were similar at 0.26, 0.25, and 0.24 g/g of consumed sugar, respectively.

The production of glycerol in YPDX medium by the three strains was similar to that in YPX medium (Figs. 2d, 3e). In contrast, MA-R4 produced a relatively high amount of acetic acid when compared with SE12 and MA-R5

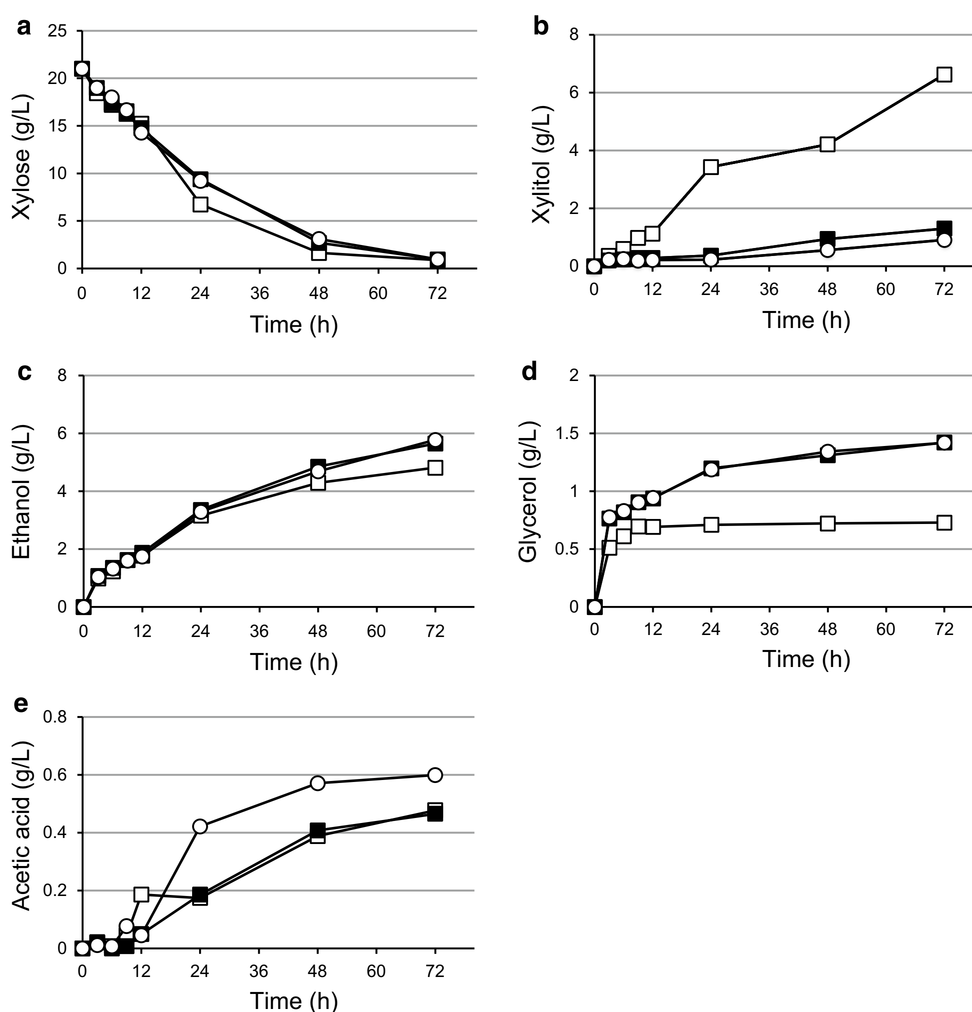

Fig. 2 Ethanol fermentation over time by the three recombinant yeast strains SE12 (*open circle*), MA-R4 (*open square*), and MA-R5 (*filled square*) in YPX medium. **a** Xylose consumption, **b** xylitol release, **c** ethanol production, **d** glycerol production, and **e** acetic acid production. Values are the average values from three independent experiments

(Fig. 3f), possibly because of the regeneration of NADPH consumed in xylose assimilation. The production of acetic acid by SE12 was significantly higher than that by MA-R5 (Fig. 3f). A similar result was obtained for acetic acid production in YPX medium (Fig. 2e). Although SE12 has an advantage with respect to reduced release of xylitol compared with MA-R5, the relatively high production of acetic acid in SE12 could potentially decrease the ethanol yield.

The similar xylose-fermenting performance in YPX and YPDX media between the SE12 and MA-R5 strains demonstrates that the haploid strains obtained in this study retain the appropriate genetic background of IR-2 for ethanol production from xylose and xylulose. The IR-2-based haploid strains are expected to construct improved xylose-fermenting diploid strains through mating with other industrial haploids. In the present study, the duplication of xylose assimilation genes by mating of recombinant haploids was found to have little effect on the improvement of ethanol fermentation from xylose (Figs. 2, 3). Kim et al. found that the improvement of xylose fermentation by mating of xylose-assimilating haploid strains was not derived from heterozygosity or genome duplication, but rather resulted from the complementation of the defective xylose-assimilation pathways between each recombinant haploid strain [20]. Kato et al. reported that improved ethanol production by xylose-assimilating mated diploid strains was obtained not only from mating but also from an increase in the copy number of the XR, XDH, and XK genes [19]. The expression levels and balance of xylose assimilation enzymes in recombinant diploid strains are important for improved ethanol fermentation from xylose [6, 16, 18, 29, 37]. Interestingly, the recombinant IR-2 with integrated pXRXDHXK or pXRARSdRXK has been reported to have higher activity of XR and XDH or ARSdR than other recombinant diploid strains with integration of the same plasmid [25, 27, 28]. These findings may explain why MA-R5 from IR-2 allowed for similar xylose fermentation to SE12 by the integration of a single set of xylose-assimilating genes.

It remains unknown whether the higher acetic acid production by SE12 is caused by the heterogeneous XDH activity. ENB3009 was found to produce a higher amount of acetic acid than MA-R4 and a recombinant strain derived

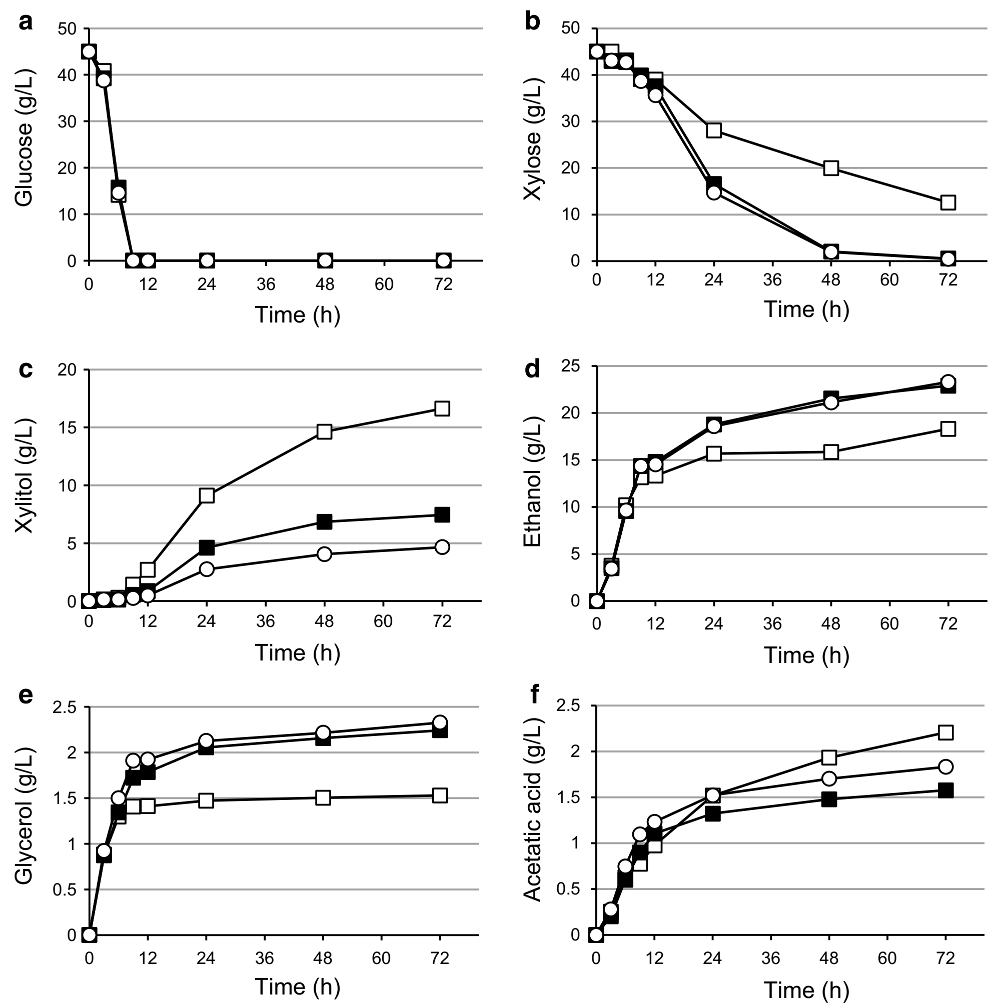

Fig. 3 Ethanol fermentation over time by the three recombinant yeast strains SE12 (*open circle*), MA-R4 (*open square*), and MA-R5 (*filled square*) in YPDX medium. **a** Glucose consumption, **b** xylose consumption, **c** xylitol release, **d** ethanol production, **e** glycerol production, and **f** acetic acid production. Values are the average values from three independent experiments

from ENB2004 with integration of pAUR-XKXDHXR in fermentation with a medium containing 50 g/L glucose and 20 g/L xylose (data not shown). This result suggests that the production of by-products for ethanol production in SE12 is related to the background of the haploid strain. The use of haploid strains having low productivity of by-products, in addition to high xylulose-fermenting ability, should be considered in future breeding of highly efficient ethanol-producing yeasts.

In conclusion, we isolated xylulose-fermentable haploid strains having the genetic background of IR-2 and found them to be useful as hosts for breeding of xylose-fermenting yeast. Characterization of the hybrid strains harboring heterogeneous XDH genes demonstrated the superiority of ARSdR ($NADP^+$-dependent XDH) in xylose assimilation; most of the selected hybrid strains showed relatively high levels of ARSdR activity, and the xylose-fermenting performance of SE12 having the same levels of ARSdR and XDH activity was very similar to that of MA-R5 having only ARSdR activity. IR-2-based haploid strains with chromosomally integrated pAUR-XKARSdRXR could thus provide diploid strains suitable for ethanol fermentation of lignocellulosic hydrolysates including xylose by heterozygous mating with haploids from robust industrial yeast having tolerance to acid and other fermentation inhibitors.

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