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Improvements in ethanol production from xylose by mating recombinant xylose-fermenting *Saccharomyces cerevisiae* strains

Hiroko Kato · Hiroaki Suyama · Ryosuke Yamada · Tomohisa Hasunuma · Akihiko Kondo

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Abstract To improve the ability of recombinant *Saccharomyces cerevisiae* strains to utilize the hemicellulose components of lignocellulosic feedstocks, the efficiency of xylose conversion to ethanol needs to be increased. In the present study, xylose-fermenting, haploid, yeast cells of the opposite mating type were hybridized to produce a diploid strain harboring two sets of xylose-assimilating genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase. The hybrid strain MN8140XX showed a 1.3- and 1.9-fold improvement in ethanol production compared to its parent strains MT8-1X405 and NBRC1440X, respectively. The rate of xylose consumption and ethanol production was also improved by the hybridization. This study revealed that the resulting improvements in fermentation ability arose due to chromosome doubling as well as the increase in the copy number of xylose assimilation genes. Moreover, compared to the parent strain, the MN8140XX strain exhibited higher ethanol production under elevated temperatures (38 °C) and acidic conditions (pH 3.8). Thus, the simple hybridization technique facilitated an increase in the xylose fermentation activity.

Keywords Xylose · Ethanol production · *Saccharomyces cerevisiae* · Diploid · Mating

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H. Kato · H. Suyama · A. Kondo (✉)
Department of Chemical Science and Engineering,
Graduate School of Engineering, Kobe University,
1-1 Rokkodaicho,
Nada, Kobe 657-8501, Japan
e-mail: akondo@kobe-u.ac.jp

R. Yamada · T. Hasunuma
Organization of Advanced Science and Technology,
Kobe University,
1-1 Rokkodaicho,
Nada, Kobe 657-8501, Japan

Introduction

Fuel ethanol produced from lignocellulosic materials by *Saccharomyces cerevisiae* is likely to become an increasingly important alternative to fossil fuel. To obtain an economically feasible industrial process for ethanol production from lignocelluloses, it is necessary to ferment all of the sugars (hexoses and pentoses) present in substrates and obtain high yields and productivities. Although *S. cerevisiae* is naturally unable to ferment pentoses, its ability to utilize xylose has been improved considerably through intensive research over the last decades (reviewed in Nevoigt 2008; van Vleet and Jeffries 2009). A key advance in these metabolic engineering studies has been the heterologous expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) derived from *Scheffersomyces* (*Pichia*) *stipitis*, along with overexpression of *S. cerevisiae* xylulokinase (XK), which are responsible for the initial steps of xylose assimilation.

However, the rate and yield of ethanol production by a variety of recombinant xylose-fermenting *S. cerevisiae* strains are significantly lower when cultured on xylose than they are on glucose. Poor xylose utilization has been attributed to potentially rate-controlling metabolic steps including the low substrate affinity of the heterologous enzymes (Kötter and Ciriacy 1993), cofactor imbalance in the XR–XDH reactions (Eliasson et al. 2000; Bruinenberg et al. 1983), and low xylose transport capacity (Runquist et al. 2009; Saloheimo et al. 2007).

One of the strategies employed to improve ethanol productivity is the use of diploid strains (Yamada et al. 2010a).

Haploid yeast cells frequently used as laboratory cells exist as one of two mating types, *MAT***a** or *MAT*α, which are specified two alternative alleles (Leu and Murray 2006). Haploid cells of the opposite mating type secrete mating pheromones which cause the cells to be propagated asexually as diploid (Elion 2000). As reported previously, compared to diploid strains, haploid strains produced in the laboratory are difficult to use for bioethanol production because they have a lower tolerance to acid, ethanol, and other fermentation inhibitors (Garay-Arroyo et al. 2004; Li et al. 2010; Martin and Jönsson 2003). However, hybridization is one of the simplest and most effective ways to improve and combine the traits of parent haploid strains (Yamada et al. 2010a). It is thus possible to generate an additive effect with respect to ethanol production from xylose through the hybridization of haploid cells of opposite mating types. In the present study, we constructed a diploid yeast strain by mating recombinant xylose-fermenting yeast strains harboring XR, XDH, and XK. Compared to the original haploid strains, the diploid strain showed more than 1.6-fold increase in ethanol production rate. Furthermore, the hybridization led to higher ethanol production under elevated temperatures and acidic conditions.

Materials and methods

Strains, plasmids, and media

Table 1 summarizes the genetic properties of all strains and plasmids used in this study. The host used for recombinant DNA manipulation was the *Escherichia coli* strain NovaBlue (Novagen, Madison, WI, USA). *E. coli* transformants were grown in Luria-Bertani medium (10 $g l^{-1}$ tryptone, 5 $g l^{-1}$ yeast extract, and 5 $g l^{-1}$ NaCl) supplemented with 100 μg ml^{-1} ampicillin. Yeast transformants and diploid strains were screened in synthetic dextrose (SD) medium [6.7 $g l^{-1}$ yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) and 20 $g l^{-1}$ glucose] supplemented with the appropriate amino acids and nucleic acids. Yeast cells were cultured aerobically in SD or yeast extract peptone dextrose (YPD; 10 $g l^{-1}$ yeast extract, 20 g l^{-1} polypeptone, and 20 $g l^{-1}$ glucose) media.

Construction of yeast strains

Plasmid pIUX1X2XK was linearized by *Eco*RV. Linearized fragments were transformed into the strains MT8-1 and NBRC1440ΔHUWL by the lithium acetate method as described previously (Katahira et al. 2006) to yield MT8-1X and NBRC1440X, respectively. Plasmid pRS405 was linearized by *Afl*II, and linearized fragments were transformed into strains MT8-1X and MT8-1 to yield MT8-1X405 and MT8-1/pRS405, respectively. The diploid strain MN8140XX was constructed by mating the haploid strains MT8-1X405 and NBRC1440X as described previously (Yamada et al. 2010b). Similarly, the diploid strains MN8140MX and MN8140NX were constructed by mating the haploid strains MT8-1X405 and NBRC1440ΔHUWL, and MT8-1/pRS405 and NBRC1440X, respectively.

Fermentation

After pre-cultivation in YPD medium for 24 h, yeast cells were cultured aerobically for 48 h at 30 °C in YPD medium. The cells were collected by centrifugation at 3,000×*g* at 4 °C for 10 min, and washed twice with distilled water. The cell pellets were then inoculated into fermentation medium [YP medium (10 $g l^{-1}$ yeast extract and 20 $g l^{-1}$ polypeptone), 50 $g l^{-1}$ xylose]. In the fermentation under acidic and high-temperature condition, 50 mM sodium citrate buffer was added into the fermentation medium to adjust pH at 3.8

Table 1 Characteristics of strains and plasmids used in this study

Strains or plasmids	Relevant features	Reference or source
Strains		
MT8-1	*MAT***a** *ade his3 leu2 trp1 ura3*	Tajima et al. (1985)
MT8-1X	MT8-1 (pIUX1X2XK)	Katahira et al. (2006)
MT8-1/pRS405	MT8-1 (pRS405)	This study
MT8-1X405	MT8-1X (pRS405)	This study
NBRC1440ΔHUWL	*MAT*α *his3 leu2 trp1 ura3*	Yamada et al. (2010b)
NBRC1440X	NBRC1440ΔHUWL (pIUX1X2XK)	This study
MN8140XX	*MAT***a**/α (2set of pIUX1X2XK and pRS405)	This study
MN8140MX	*MAT***a**/α (1set of pIUX1X2XK and pRS405)	This study
MN8140NX	*MAT***a**/α (1set of pIUX1X2XK and pRS405)	This study
Plasmids		
pRS405	*LEU2*	Stratagene, CA, USA
pIUX1X2XK	*URA3*, expression of *Xyl1*, *Xyl2*, and *Xks1* genes	Katahira et al. (2006)

and 5.5, respectively. The initial cell concentration was set at 50 g of wet cells per liter. Fermentations were performed under oxygen-limited conditions in 50-ml cultures in 100-ml glass bottles at 30 °C using magnetic stirrer at 500 rpm. For determining the concentration of ethanol, xylitol, xylose, and glycerol in the fermentation medium, the supernatant obtained by centrifugation at 6,000×*g* at 4 °C for 5 min was applied to high-performance liquid chromatography (HPLC). A Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan) was used with an RID-10A refractive index detector (Shimadzu). The HPLC system was operated at 80 °C with water at a flow rate of 0.6 ml min^{-1} as the mobile phase.

Analysis of intracellular metabolites

Yeast cells in 5-ml fermentation medium were harvested by centrifugation at 1,000×*g* at 4 °C for 5 min after 30 h fermentation. Metabolites were extracted using a modification of a previously described method (Yoshida et al. 2008). Briefly, the cell pellet was washed twice with cold water to remove the fermentation medium and chilled on ice. The harvested cells were then immediately frozen in liquid nitrogen and lyophilized. The dried cells (10 mg) were then homogenized using a Shake Master NEO (Bio Medical Science, Tokyo, Japan) at 1,500 rpm at 4 °C for 5 min with 300 mg of zirconia silica beads (0.6 mm) and one silica ball (5 mm) in 900 ml of solvent mixture ($CHCl_3$:CH_3OH:H_2O, 1:2.5:1, *v*/*v*/*v*) containing 356 mM adipic acid and 3.56 mM PIPES as internal standards for semi-quantitative analysis. The samples were then shaken at 1,200 rpm at 15 °C for 30 min and centrifuged at 15,000 rpm at 4 °C for 3 min. A 630-ml aliquot of the supernatant was then transferred to an Eppendorf tube and mixed well after the addition of 280 ml water. After centrifugation at 15,000 rpm at 4 °C for 3 min, 300 ml of the upper phase was transferred to a new tube and vacuum-dried. Quantitative analysis of 47 intracellular metabolites was performed by gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) as described previously (Hasunuma et al. 2011). Ionic metabolites such as sugar phosphates [dihydroxyacetone phosphate (DHAP), erythrose-4-phosphate (E4P), glyceraldehyde-3-phosphate (GAP), phosphoenolpyruvate (PEP), 2-phosphoglycerate (2PGA), 3-phosphoglycerate (3PGA), and sedoheptulose-7-phosphate (S7P)], organic acids (citrate, fumarate, *iso*-citrate, 2-ketoglutarate, malate, lactate, pyruvate, and succinate), nucleotides (ADP and ATP), and coenzymes (CoA, acetyl-CoA, NAD^+, NADH, $NADP^+$, and NADPH) were analyzed by CE-MS. Amino acids, phosphate, sugars and sugar alcohols, and uracil were quantified by GC-MS. Commercially available standard compounds were analyzed in parallel with the samples. The mass spectra and retention time obtained were used to identify the metabolites. Metabolite concentrations were calculated by the peak area after normalization with the peak area of internal standards. The quantitative data of metabolites (acetyl-CoA, alanine, β-alanine, arginine, asparagine, aspartic acid, ADP, ATP, citrate, DHAP, E4P, fumarate, F6P, GAP, glutamic acid, glutamine, glycerol, G6P, histidine, *iso*-citrate, *iso*-leucine, 2-ketoglutarate, lactate, leucine, lysine, malate, NAD^+, NADH, $NADP^+$, NADPH, ornithine, PEP, 2PGA, 3PGA, phenylalanine, phosphate, proline, pyruvate, ribitol, serine, S7P, succinate, threonine, trehalose, tyrosine, uracil, valine, xylitol, and xylose) was subjected into primary component analysis (PCA) after pretreatment by mean centering with the commercial software Pirouette (GL Science, Tokyo, Japan). It created the secondary dimension score plots to visualize the contrast between different yeast samples. The reason for the clusters separation was indicated by the loading plots of the corresponding principal component.

Results

Mating of xylose-fermenting yeast strains

The diploid strain MN8140XX containing two sets of xylose assimilation genes was constructed by mating the recombinant xylose-fermenting haploid strains MT8-1X and NBRC1440X. The haploid strains MT8-1X and NBRC1440X were generated by the chromosomal integration of pIUX1X2XK into the *URA3* locus in MT8-1 and NBRC1440ΔHUWL to express the XR and XDH genes derived from *S. stipitis*, and the endogenous XK gene under the control of the *TDH3* promoter (Fig. 1). The MT8-1X405 strain, which lost auxotrophy for leucine, was constructed by chromosomal integration of plasmid pRS405 to the *LEU2* locus in MT8-1X. Then, MT8-1X405 and NBRC1440X were hybridized to yield the diploid MN8140XX strain, which was selected on the basis of auxotrophy for both histidine and tryptophan.

Effects of hybridization of recombinant xylose-fermenting haploid strains on xylose fermentation

Xylose fermentation ability under oxygen-limited conditions at 30 °C was examined in the diploid strain MN8140XX and its parent strains MT8-1X405 and NBRC1440X in YP media containing 50 gl^{-1} xylose as a sole carbon source. As shown in Fig. 2, the diploid strain MN8140XX, which contains two sets of xylose assimilation genes, demonstrated higher fermentation ability compared to both of its parent strains, MT8-1X405 and NBRC1440X, where each of its parent strains had one set of xylose-assimilating genes. The hybrid strain MN8140XX had a xylose consumption rate that was 1.6- and 3.4-fold higher (2.73 gl^{-1} h^{-1}) than MT8-1X405 and

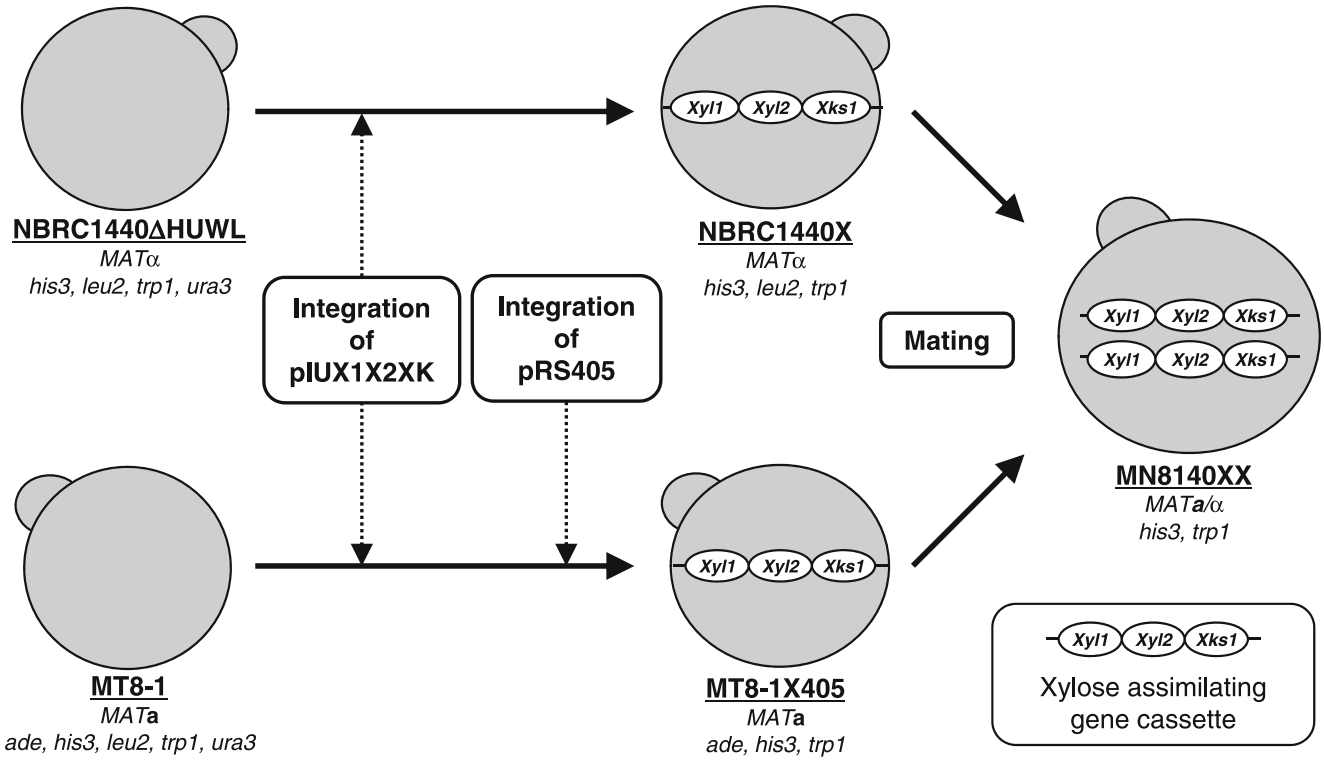

Fig. 1 Strategy employed for constructing diploid yeast strains

NBRC1440X, respectively (Table 2). The ethanol production rate of MN8140XX was 0.89 gl^{-1} h^{-1}, which was 1.6- and 2.8-fold higher than that obtained from MT8-1X405 and NBRC1440X, respectively (Table 2). The ethanol produced

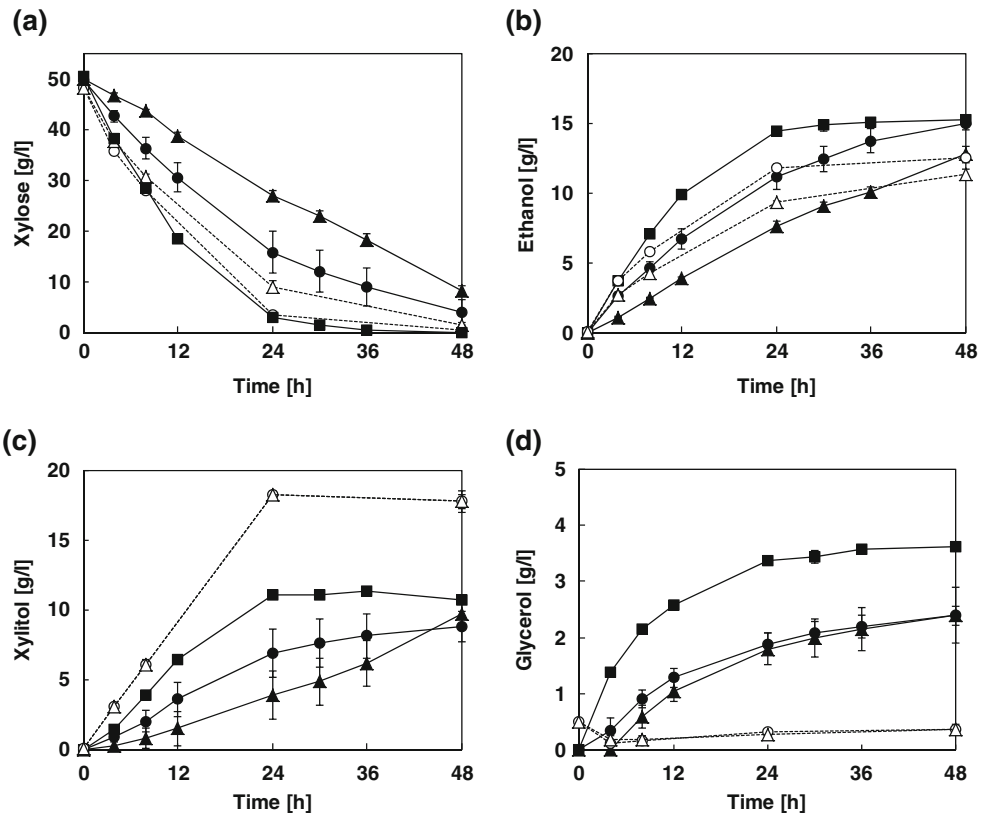

Fig. 2 Time course of **a** xylose utilization, and **b** ethanol, **c** xylitol, and **d** glycerol production associated with xylose fermentation using MT8-1X405 (*closed circles*), NBRC1440X (*closed triangles*), MN8140XX (*closed squares*), and MN8140MX (*open circles*), and MN8140NX (*open triangles*) at 30 °C. Data points are averages of results obtained from four independent experiments. *Bars* show standard errors of the averages

Table 2 Fermentation performance of haploid and diploid yeast strains

Strain	Xylose consumption rate (g-xylose l^{-1} h^{-1})	Ethanol production rate (g-ethanol l^{-1} h^{-1})
MT8-1X405	1.68±0.28	0.57±0.06
NBRC1440X	0.80±0.03	0.32±0.01
MN8140XX	2.73±0.06	0.89±0.02
MN8140MX	2.53±0.08	0.71±0.01
MN8140NX	2.17±0.05	0.52±0.04

Values are the averages of the results from four independent experiments ± SEM

by MN8140XX after 24 h fermentation was 14.5±0.19 gl^{-1}, which was 1.3- and 1.9-fold higher than that obtained from MT8-1X405 (11.2±0.92 gl^{-1}) and NBRC1440X (7.7±0.32 g l^{-1}), respectively. The cell concentrations of all strains were constant during the batch fermentation (data not shown). Since pH was not adjusted during fermentation, the pH of the fermentation medium was 6.1 at the start of the fermentation and 5.0 from 12 to 48 h after starting the fermentation.

Effect of increasing the number of xylose-assimilating gene cassettes on xylose fermentation

In order to evaluate the effect of increasing the number of xylose-assimilating gene cassettes (Fig. 1) on ethanol production from xylose, the diploid strains MN8140MX and MN8140NX, each of which contain one set of xylose-assimilating genes ,were constructed by hybridizing NBRC1440ΔHUWL and MT8-1X405, and NBRC1440X and MT8-1/pRS405, respectively.

The xylose consumption rates of MN8140MX and MN8140NX were 92% and 79%, respectively, which is lower than that of MN8140XX (Table 2). The ethanol production rates of MN8140MX and MN8140NX were 80% and 58% lower than that of MN8140XX, respectively (Table 2). After 24 h fermentation, the ethanol production of MN8140MX and MN8140NX was 11.8±0.11 and 9.4±0.11 gl^{-1}, which was lower than that of MN8140XX (14.5±0.19 gl^{-1}; Fig. 2b). As shown in Fig. 2c, xylitol production by MN8140MX and MN8140NX was higher than that of MN8140XX (Fig. 2c), but glycerol production by MN8140MX and MN8140NX were markedly lower than MN8140XX (Fig. 2d).

Comparison between diploid strains harboring one xylose-assimilating gene cassette and haploid xylose-fermenting strains

The effect of chromosome doubling on ethanol production from xylose was evaluated. The ethanol fermentation ability of strains MN8140MX and MN8140NX, each of which contain one set of xylose-assimilating genes, was compared against that of the parental xylose-utilizing strains MT8-1X405 and NBRC1440X, each of which also contained one set of xylose-assimilating genes. The xylose consumption rate of MN8140MX (2.53±0.08 gl^{-1} h^{-1}) was higher than that of MT8-1X405 (1.68±0.28 gl^{-1} h^{-1}; Fig. 2). In addition, MN8140NX showed a higher xylose consumption rate (2.17±0.05 gl^{-1} h^{-1}) than NBRC1440X (0.80±0.03 g l^{-1} h^{-1}; Table 2). Ethanol production rates of MN8140MX and MN8140NX were higher than those of their parent xylose-utilizing strains. These results indicate that the hybridization of haploid strains improves xylose utilization regardless of the gene copy number of XR, XDH, and XK; however, an increase in the copy number of these genes also improves ethanol production from xylose (Fig. 2).

MT8-1X405 had a higher xylose consumption rate, ethanol production rate, and produced more ethanol than NBRC1440X after 24 h fermentation (Table 2 and Fig. 2b). After 24 h fermentation, MN8140MX, which was generated by MT8-1X405, had a higher xylose consumption rate, ethanol production rate, and produced more ethanol than MN8140NX, which was generated by NBRC1440X (Table 2 and Fig. 2b). These increases may be attributed to the inheritance of the xylose fermentation ability of the parent strains.

Enhancement of ethanol production under acidic conditions by crossbreeding

In order to evaluate the xylose fermentation profiles of recombinant strains under acidic conditions, xylose fermentation was performed at pH 3.8 (Fig. 3a). Applied under the acidic condition, the haploid strain NBRC1440X demonstrated 61% and 70% decrease in xylose consumption rate and ethanol production rate, respectively. Specifically, after 24 h fermentation, ethanol production by NBRC1440X was 5.56 gl^{-1} at pH 3.8, while it was 7.68 gl^{-1} under no pH-control condition (Figs. 2b and 3a). These findings indicate that NBRC1440X is sensitive to acidic conditions, which reduce the ethanol production ability of NBRC1440X. Conversely, after 24 h fermentation, MN8140XX produced 14.51 gl^{-1} ethanol at pH 3.8 and 14.65 gl^{-1} under no pH-control condition (Figs. 2b and 3a). Taken together, these findings indicate that ethanol production under acidic conditions was improved by crossbreeding. Although MT8-1X405 showed higher ethanol production even at pH 3.8, the amount of ethanol produced by MN8140XX was higher than that of its haploid parent strains under acidic conditions (Figs. 2b and 3a).

Enhancement of ethanol production under high-temperature conditions by crossbreeding

For the evaluation of xylose fermentation ability at elevated temperature, xylose fermentation using recombinant strains

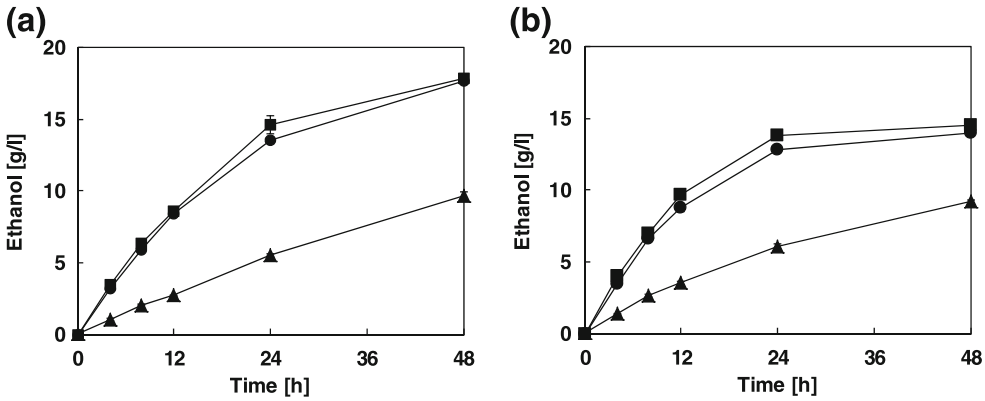

Fig. 3 Change in ethanol concentration in xylose fermentation under **a** pH 3.8 at 30 °C and **b** under pH 5.5 at 38 °C by haploid strains MT8-1X405 (*circles*), NBRC1440X (*triangles*), and the diploid strain MN8140XX (*squares*). Data points are averages of results obtained from four independent experiments. *Bars* show standard errors of the averages

was performed at 38 °C. As shown in Figs. 2b and 3b, increasing the temperature from 30 °C to 38 °C resulted in a decrease in ethanol production by the haploid strain NBRC1440X, indicating that NBRC1440X was sensitive to elevated temperatures. However, ethanol production by the diploid strain MN8140XX at 38 °C was similar to that produced at 30 °C after fermentation for 24 h (Figs. 2b and 3c). Indeed, even at 38 °C, MN8140XX (13.79 $g l^{-1}$) produced more ethanol than its parent strains after 24 h.

Metabolite profiling of xylose-fermenting yeast strains

In the present study, hybridization technique facilitated an increase in the xylose fermentation activity, which should be due to chromosome doubling as well as the increase in the copy number of xylose assimilation genes. However, metabolic features that characterize xylose fermentation ability of haploid and diploid were not elucidated. Deciphering the metabolomic distinction of diploid and haploid yeasts would lead to a better understanding of them. Thus, we comprehensively determined the intracellular metabolites in haploid and diploid strains used for xylose fermentation at 30 °C under oxygen-limited condition. The metabolic variations of haploid and diploid were evaluated by GC-MS and CE-MS combined with statistical analysis. After the intracellular metabolites were identified, a PCA based on metabolite accumulation levels was performed. The score plot of the PCA (Fig. 4) exhibited the similarities and differences between samples, with primary components (PC) 1 and 2 selected to visualize and distinguish between the metabolic phenotypes. PC1 included 90.3% of the total information derived from metabolite variances, and PC2 contained 6.7%. This result indicates that the diploid strain MN8140XX showed metabolic phenotypes that were distinct from the haploid parent strains MT8-1X405 and NBRC1440X. The score plots obtained for PC1 of the diploid strain were located closer to that of NBRC1440X rather than MT8-1X405, suggesting that MN8140X might inherit metabolic characteristics of NBRC1440X. Despite the auxotrophy, the origin of the parent strains is different. A laboratory strain, MT8-1 (*MAT***a**, *ade his3 leu2 trp1 ura3*), is a meiotic segregant of a diploid strain (Tajima et al. 1985), while NBRC1440, which is origin of NBRC1440ΔHUWL (*MATα*, *his3 leu2 trp1 ura3*), is a deposited strain (Yamada et al. 2010b). It is noteworthy to mention that MN8140XX resembles NBRC1440X as they both exhibit flocculation properties. The loading plot of PC1 shows significant metabolites that influence the separation between MT8-1X405, NBRC1440X, and MN8140XX (Supplementary Fig. S1). The intracellular content of xylose, xylitol, and amino acids such as alanine, histidine, lysine, arginine, glutamic acid, proline, and β-alanine contributed to the separation by PC1, which was considered as strains-specific behavior.

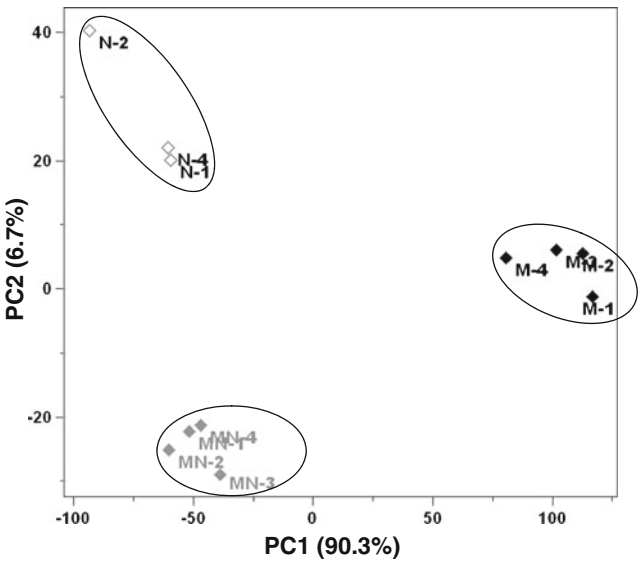

Fig. 4 PCA score plot of metabolite profiling. Metabolite profiling data of 4 four clones of MT8-1X405 (*black*,; *M-1*, *M-2*, *M-3*, and *M-4*), 3 three clones of NBRC1440X (*white*,; *N-1*, *N-2*, and *N-4*), and 4 four clones of MN8140XX (*gray*,; *MN-1*, *MN-2*, *MN-3*, and *MN-4*) was applied to the PCA. Primary components (PC) 1 and 2 were selected because they best illustrated the distinction between metabolic phenotypes and include 96.9% of the total information content derived from the observed metabolite variances

Discussion

We demonstrated that the hybridization of recombinant XR/XDH/XK-based xylose-fermenting *S. cerevisiae* strains resulted in improved ethanol production from xylose. At 30 °C, the hybrid strain MN8140XX showed a 1.3- and 1.9-fold increase in ethanol production compared to its parent strains MT8-1X405 and NBRC1440X, respectively (Fig. 2). In addition, the xylose consumption rate of the hybrid was 1.6- and 2.8-fold higher than that of MT8-1X405 and NBRC1440X, respectively. Thus, the effect of the hybridization technique clearly enhanced the xylose fermentation activity of the hybrid compared to its haploid parents.

Compared to its parent strains, the diploid MN8140XX strain also showed higher ethanol production under elevated temperatures (38 °C) and acidic conditions (pH 3.8; Fig. 3). Although the parent strain NBRC1440X showed lower rates of ethanol production and xylose consumption than the other parent strain MT8-1X405, the resulting diploid strain MN8140XX, obtained by hybridizing NBRC1440X and MT8-1X405, showed higher ethanol production than the more productive haploid strain, MT8-1X405. Thus, the hybridization strategy is a promising method for improving ethanol production under conditions that typically inhibit fermentation. Ethanol production at high temperatures has recently received increased attention because cooling is expensive (Fonseca et al. 2008). Other advantages of elevated temperature tolerance include efficient simultaneous saccharification and fermentation and continuous ethanol removal during industrial bioethanol production (Banat et al. 1998). In addition, ethanol fermentation under acidic conditions has the advantage of reducing bacterial contamination. In the present study, MN8140XX showed higher cell growth in YPD medium under aerobic condition than its parent strains (data not shown) demonstrating that hybridization is one of the most effective methods for improving and combining traits of haploid parent strains for the industrial purposes.

As reported previously, compared to haploid strains, industrial polyploid strains, including diploid strains, have higher cell growth rates, cell yields, and tolerances to various stresses such as heat shock, hyperosmosis, high reactive oxygen species level, and inhibitor stress (Garay-Arroyo et al. 2004; Hashimoto et al. 2006; Higgins et al. 2001; Martin and Jönsson 2003). Garay-Arroyo et al. (2004) reported higher resistance of industrial strains to heat shock and to an oxidative environment than haploid laboratory strains. A similar behavior was observed regarding ethanol production from glucose in the presence of toxins such as furfural and acetic acid. However, the possible mechanism of the alleviation of such stresses by using diploid strains remains unclear. No significant correlation between the stress tolerance of the strains and the transcript level of stress-responsive genes was found (Garay-Arroyo et al. 2004). In the present study, we first reported the improvement of ethanol production from xylose under acidic and high-temperature condition by using diploid strains, while the mechanism of tolerance to such stress conditions should be analyzed in further studies.

The diploid strains MN8140MX and MN8140NX obtained by hybridization of a recombinant xylose-fermenting strain (MT8-1X405 or NBRC1440X) and a wild-type strain (NBRC1440ΔHUWL or MT8-1) showed higher ethanol production rates than their recombinant haploid parent strains. On the other hand, ethanol production by diploid strains harboring one copy of the XR/XDH/XK gene cassette was lower than that of the two-copy strain MN8140XX. These results indicate that the improved ethanol production was obtained not only from hybridization but also from an increase in copy number of XR/XDH/XK genes. As shown in Table 2, ethanol production rate of MN8140MX was higher than that of MN8140NX. Since the haploid strain MT8-1X demonstrated better fermentation ability than NBRC1440X, the trait of relatively high ethanol production in MN8140X may be derived from the parent strain MT8-1X.

Recently, comprehensive biological technologies such as metabolomics, transcriptomics, and proteomics have been used for systematically comparing metabolic features of diploid and haploid yeast to provide insights into difference of haploid and diploid yeast in pheromone pathway and ethanol tolerance mechanism (de Godoy et al. 2008; Ding et al. 2010; Li et al. 2010). This study revealed that metabolite profiles originating from three strains (MN8140XX, MT8-1X405, and NBRC1440X) were distinctive and distinguished by PCA (Fig. 4). According to the metabolic profiling, MN8140X has more similar metabolic features to NBRC1440X than MT8-1X405, while the diploid strain located between the two parent strains in PC1 that included more than 90% of the total metabolite information. This result suggests that MN8140X might inherit metabolic characteristics of NBRC1440X. To our knowledge, this is the first report of metabolome analysis of haploid versus diploid xylose-fermenting recombinant strains. The loading plot of PC1 indicated that the difference of intracellular accumulation of amino acids as well as metabolites involved in *S. stipitis* pathway such as xylose and xylitol strongly contributed to the discrimination of three strains. Accumulation of these compounds might affect ethanol productivity from xylose.

Xylose fermentation has been performed using hybrid strains produced by protoplast fusion between *S. cerevisiae* and natural xylose-fermenting yeasts (Pasha et al. 2007; Yan et al. 2009). Although *Candida shehatae*, *S. stipitis*, and *Pachysolen tannophilus* are the best native xylose-fermenting

yeasts, they have a relatively low ethanol tolerance. Consequently, protoplast fusion of *S. cerevisiae* and such xylose-fermenting yeasts yielded fusant strains that were capable of utilizing xylose and producing ethanol. However, the efficiency of heterocellular fusion was lower than that of homologous cell hybridization, and the genetic stability of the fusants over 15 generations is still unclear. The relatively lower stability is partly because protoplast fusion is a non-specific recombination technique used for transferring cytosolic organelles and genetic material.

In the present study, we demonstrated that the hybridization of xylose-fermenting *S. cerevisiae* strains is a simple and effective way to improve ethanol production. In general, compared to industrial diploid or polyploid strains, the practical application of laboratory haploid strains to bioethanol production is complicated by their relatively low tolerance to acid, ethanol, and other fermentation inhibitors contained in lignocellulosic hydrolysate (Garay-Arroyo et al. 2004; Martin and Jönsson 2003). The hybridization strategy is thus considered to be well suited for use in industrial ethanol production from lignocellulosic hydrolysates.

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References

Banat IM, Nigam P, Singh D, Marchant R, McHale AP (1998) Ethanol production at elevated temperatures and alcohol concentrations: Part I—yeasts in general. World J Microbiol Biotechnol 14:809–821

Bruinenberg PM, Debot PH, van Dijken JP, Scheffers WA (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur J Appl Microbiol Biotechnol 18:287–292

de Godoy LMF, Olsen JV, Cox J, Nielsen ML, Hubner NC, Fröhlich F, Walther TC, Mann M (2008) Comprehensive mass-spectrometry based proteome quantification of haploid versus diploid yeast. Nature 455:1251–1255

Ding MZ, Li BZ, Cheng JS, Yuan YJ (2010) Metabolome analysis of differential responses of diploid and haploid yeast to ethanol stress. OMICS 14:553–561

Eliasson A, Christensson C, Wahlbom CF, Hahn-Hägerdal B (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium. Appl Environ Microbiol 66:3381–3386

Elion EA (2000) Pheromone response, mating and cell biology. Curr Opin Microbiol 3:573–581

Fonseca GG, Heinzle E, Wittmann C, Gombert AK (2008) The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol 79:339–354

Garay-Arroyo A, Covarrubias AA, Clark I, Niño I, Gosset G, Martinez A (2004) Response to different environmental stress conditions of industrial and laboratory *Saccharomyces cerevisiae* strains. Appl Microbiol Biotechnol 63:734–741

Hashimoto S, Aritomi K, Minohara T, Nishizawa Y, Hoshida H, Kashiwagi S, Akada R (2006) Direct mating between diploid sake strains of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 69:689–696

Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A (2011) Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. Microb Cell Fact 10:2

Higgins VJ, Bell PJL, Dawes IW, Attfield PA (2001) Generation of a novel *Saccharomyces cerevisiae* strain that exhibits strong maltose utilization and hyperosmotic resistance using nonrecombinant techniques. Appl Environ Microbiol 67:4346–4348

Katahira S, Mizuike A, Fukuda H, Kondo A (2006) Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cellooligosaccharide-assimilating yeast strain. Appl Microbiol Biotechnol 72:1136–1143

Kötter P, Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776–783

Leu JY, Murray AW (2006) Experimental evolution of mating discrimination in budding yeast. Curr Biol 16:280–286

Li BZ, Cheng JS, Ding MZ, Yuan YJ (2010) Transcriptome analysis of differential responses of diploid and haploid yeast to ethanol stress. J Biotechnol 148:194–203

Martin C, Jönsson LJ (2003) Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. Enzyme Microb Technol 32:386–395

Nevoigt E (2008) Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 72:379–412

Pasha C, Kuhad RC, Rao LV (2007) Strain improvement of thermotolerant *Saccharomyces cerevisiae* VS3 strain for better utilization of lignocellulosic substrates. J Appl Microbiol 103:1480–1489

Runquist D, Fonseca C, Radstrom P, Spencer-Martins I, Hahn-Hägerdal B (2009) Expression of Gxf1 transporter from *Candida intermedia* improves fermentation performance in recombinant xylose-utilizing *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 82:123–130

Saloheimo A, Rauta J, Stasyk OV, Sibirny AA, Pentillä M, Ruohonen L (2007) Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. Appl Microbiol Biotechnol 70:6816–6825

Tajima M, Nogi Y, Fukasawa T (1985) Primary structure of the *Saccharomyces cerevisiae* GAL7 gene. Yeast 1:67–77

van Vleet JH, Jeffries TW (2009) Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 20:300–306

Yamada R, Tanaka T, Ogino C, Kondo A (2010a) Gene copy number and polyploidy on products formation in yeast. Appl Microbiol Biotechnol 88:849–857

Yamada R, Tanaka T, Ogino C, Kondo A (2010b) Novel strategy for yeast construction using δ-integration and cell fusion to efficiently produced ethanol from raw starch. Appl Microbiol Biotechnol 85:1491–1498

Yan F, Bai F, Tian S, Zhang J, Zhang Z, Yang X (2009) Strain construction for ethanol production from dilute-acid lignocellulosic hydrolysate. Appl Biochem Biotechnol 157:473–482

Yoshida S, Imoto J, Minato T, Oouchi R, Sugihara M, Imai T, Ishiguro T, Mizutani S, Tomita M, Soga T, Yoshimoto H (2008) Development of bottom-fermenting *Saccharomyces* strains that produce high SO_2 levels, using integrated metabolome and transcriptome analysis. Appl Environ Microbiol 74:2787–2796