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A UV-induced mutant of *Pichia stipitis* with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance

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ABSTRACT

In the fermentation process of lignocellulosic biomass (such as wood and rice straw), efficient conversion of pentose (mainly xylose) into ethanol is important. Mutants of *Pichia stipitis* NBRC1687 were obtained after UV mutagenesis and selection of large colonies on ethanol-containing medium. One mutant, PXF58, produced 4.3% ethanol from 11.4% xylose while the parent strain only produced 3.1%. The ethanol productivities of PXF58 from glucose and fructose were about were about 1.4-fold higher than those of the parent strain. After continuous cultivation of PXF58 in YNB (yeast nitrogen base) medium containing 2% xylose and 5–7% ethanol, an ethanol-tolerant mutant, PET41, was obtained. Strain PET41 was able to produce 4.4% ethanol when first supplied with xylose then with glucose. This isolate might be thus useful for two-phase fermentation in which xylan is saccharified by xylanase to produce xylose, and glucan is saccharified later by cellulase and β-glucosidase to produce glucose.

1. Introduction

Xylose is the second most abundant fermentable sugar in the hydrolysate of lignocellulosic biomass (such as wood and rice straw). Except for genetically engineered strains (Deng and Ho, 1990), the yeast most commonly used for ethanol production, *Saccharomyces cerevisiae,* cannot ferment xylose. *Pichia stipitis* (Ferrari et al., 1992), *Candida shehatae* (Wayman and Parekh, 1985), and *Pachysolen tannophilus* (Schneider et al., 1981) are able to produce ethanol, but very slowly and at low concentrations. For the efficient production of ethanol from xylose, microaerophilic conditions are important to maintain cell viability and NADH balance (Afbogbo et al., 2006). Furthermore, these yeasts show low tolerance to ethanol (Barbosa et al., 1990) and the inhibitory by-products of lignocellulosic hydrolysates (Lohmeier-Vogel et al., 1998).

Although the introduction of the xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *P. stipitis* into *S. cerevisiae* strains enabled production of ethanol from xylose (Wahlbom et al., 2003a,b; Chu and Lee, 2007), these recombinant strains produced xylitol in addition to ethanol (Deng and Ho, 1990; Matsushika et al., 2008). Matsushika et al. (2009) created recombinant *S. cerevisiae* stains that efficiently produced ethanol from lignocellulosic eucalyptus-based hydrolysate without glucose repression of xylose utilization. However, the application of recombinant strains at industrial plants in Japan is difficult because of the costs of measures to prevent the accidental release of the genetically engineered microorganisms and the need to perform environmental assessments.

Chiung-Fang et al. (2009) attempted to improve ethanol yields of *Pichia stipites* by adapting the strain to the fermentation inhibitors present in acid-hydrolyzed rice straw, and Bajwa et al. (2009) obtained a strain that was adapted to spent hardwood sulfite liquor after UV mutatagenesis. These strains produced ethanol from the lignocellulosic hydrolysates more rapidly than the parent strains in the presence of inhibitors, but ethanol productivities in the absence of the inhibitors were not improved.

Changes to the ethanol production processes were also explored to improve ethanol production from lignocellulosic hydrolysate. Li et al. (2010) developed two-phase simultaneous saccharification and fermentation (TPSSF) of corn stover-derived xylose and glucose. In the TPSSF, xylan was saccharified by xylanase to produce xylose in the first phase, and glucan was saccharified by cellulase and β-glucosidase to produce glucose in the second phase. The xylose-fermenting recombinant *Escherichia coli* and *S. cerevisiae* were used for xylose fermentation in the first phase and glucose fermentation in the second phase, respectively. Overall, the TPSSF using aqueous ammonia pretreated corn stover, 84% of the theoretical ethanol yield was obtained. The two-phase process is an efficient method of avoiding glucose repression.

In the present study, we attempted to improve the ethanol productivity of xylose-fermenting *Pichia stipitis* by UV-mutagenesis and by selection of mutants with increased ethanol tolerance. The mutants were tested in a two-phase fermentation process similar to that used for TPSSF.

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2. Methods

2.1. Strains and media

P. stipitis NBRC1687 (NRRL Y-7124, ATCC58376), NBRC10063, *P. tannophilus* NBRC1007 and *C. shehatae* NBRC1983 (NRRL Y-12858, ATCC34887) were obtained from the culture collection of the NITE Biological Resource Center (NBRC, Japan). *C. shehatae* JCM9837 and JCM9842 were obtained from the Japan collection of microorganisms (JCM) of the Riken Bioresource Center (Japan). *Kluyveromyces marxianus* NFRI3803, NFRI3842, NFRI3844 and NFRI3860 were obtained from the Microbiological Bank at our institute (NFRI). *P. stipitis* DY4, *Candida tropicalis* KXF3, KXF4, and KXF5, *Candida novaki* TXF21 and TXF44, *Candida* sp. TXF2–4, *Candida quercuum* IXF5, and *Candida solani* IXF6 and IXF7 were isolated from soil and rotted-wood samples collected at Tsukuba city in Japan. *P. stipitis* PXF4, PXF36, and PXF58 were derived from *P. stipitis* NBRC1687. These strains were maintained on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar).

YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for pre-cultivation. Fermentation media (YNBX) containing 0.17% yeast nitrogen base (YNB, without amino acids and ammonium sulfate), 0.2% ammonium sulfate, 0.5% peptone, and 2–12% xylose were used for xylose fermentation experiments. Glucose and fructose (12%) were also used for investigations of fermentation capability. Screening plates containing 0.17% YNB (without amino acids and ammonium sulfate), 0.2% ammonium sulfate, 0.5% peptone, 5% xylose, 5% ethanol, and 2% agar were used for selection of yeasts with increased xylose-fermentation capability.

2.2. UV mutation

Yeast strains were cultivated in 5 ml of YPD medium with shaking for 24 h. The culture (1 ml) and 19 ml of sterilized water were mixed in plastic petri dishes (OD_{600} was around 1). The mixture was stirred with a magnetic stirrer and exposed to UV light at a distance of 50 cm for 30 s. One hundred μl of the UV-irradiated mixture was diluted to 5 ml with fermentation medium containing 5% xylose and cultivated with shaking for 24 h. After 10^5-fold dilution with sterilized water, the diluted culture was spread on screening plates. Following overnight cultivation, about 500 colonies were apparent on the plates.

2.3. Analytical methods

Ethanol, xylose, glucose, and fructose concentrations were analyzed using high-performance liquid chromatography (Shimadzu HPLC LC20AD, Shimadzu, Japan) with an RI detector and a fermentation monitoring column (Bio-Rad Laboratories, Hercules, CA, USA). The analytical conditions have been described by Watanabe et al. (2008).

The ethanol productivity was calculated using the following equation (Kida et al., 1992):

$$\text{Productivity}(g/\text{Lh}) = (P_f - P_i)/t \quad (1)$$

where P_i is the initial ethanol concentration (g/L), P_f is the final ethanol concentration (g/L), and t is the fermentation time (h).

The maximum theoretical ethanol yield from sugar (glucose, fructose, xylose) was calculated using the following stoichiometric relation: 100 g of sugar produces 51.1 g of ethanol and 48.9 g of CO_2. Ethanol yields were calculated using the following equations:

$$\text{Yield}(\text{from hexose})(\%) = (P_f - P_i)/(S_i - S_f) \times 100/0.511 \quad (2)$$

where P_i is the initial ethanol concentration (g/L), P_f is the final ethanol concentration (g/L), S_i is the initial sugar concentration (g/L), and S_f is the final sugar concentration (g/L).

The absorbance at 660 nm (OD_{660}) was measured in order to determine cell density. Total cells number was calculated using the relation of OD_{660} and total cells number counted using a hemacytometer. The number of viable yeast cells was determined using the YM plate medium. The viability was represented by the ratio of the number of viable cells to the number of total cells.

2.4. Fermentation process

Yeast strains were pre-cultivated with shaking in 5 mL of YPD medium in tubes at 30 °C, 150 rpm for 24 h. Fermentation medium (45 mL) and 5 mL of pre-culture were poured in a 100-mL flask and incubated in an incubator (BCP-320F; TAITEC, Sai tama, Japan) with shaking at 120 rpm under microaerobic conditions (closed with a silicon plug). Aliquots (1 mL) were centrifuged at 7600*g* for 1 min and xylose and ethanol concentrations in the supernatant were determined.

2.5. Two-phase fermentation

Two-phase fermentation was performed using 40 mL of fermentation medium containing 4.5% xylose and 5 mL of pre-culture in 100-mL flasks and incubation at 30 °C with shaking at 120 rpm under microaerobic conditions. Two and 3 mL of glucose (80% w/v) was added after 36 and 60 h, respectively.

3. Results and discussion

3.1. Selection of enhanced xylose-fermenting mutants

Among the 20 yeast strains tested, *P. stipitis* NBRC1687 produced ethanol the fastest (Table 1), and this strain was subjected to UV-mutagenesis. Of 66 colonies that grew larger than wild-type colonies on YNBX 5% ethanol plates, three (PXF4, PXF36, and PXF58) showed more efficient ethanol production than the parental (Table 2) even after several cycles of growth on YPD plates. Among these mutants, PXF58 produced ethanol from xylose with the highest yield. PXF58 also produced ethanol most rapidly (data not shown).

Table 1
Xylose fermentations of 20 xylose-fermenting yeast strains.

Strain		Residual Xylose (%)	Ethanol (%)
Blank		4.98 ± 0.07	–
Pichia stipitis	NBRC1687	0.97 ± 0.28	1.47 ± 0.11
Pichia stipitis	NBRC10063	1.10 ± 0.12	1.35 ± 0.06
Pichia stipitis	DY4	1.27 ± 0.22	1.26 ± 0.08
Candida shehatae	NBRC1983	1.05 ± 0.32	0.99 ± 0.17
Candida shehatae	JCM9837	1.54 ± 0.23	0.90 ± 0.09
Candida shehatae	JCM9842	2.27 ± 0.21	0.82 ± 0.07
Pachysolen tannophilus	NBRC1007	2.41 ± 0.31	0.64 ± 0.14
Kluyveromyces marxianus	NFRI3803	3.63 ± 0.22	0.08 ± 0.00
Kluyveromyces marxianus	NFRI3842	3.71 ± 0.11	0.12 ± 0.01
Kluyveromyces marxianus	NFRI3844	3.92 ± 0.07	0.10 ± 0.01
Kluyveromyces marxianus	NFRI3860	3.98 ± 0.02	0.01 ± 0.00
Candida tropicalis	KXF3	2.78 ± 0.19	0.06 ± 0.00
Candida tropicalis	KXF4	2.62 ± 0.17	0.06 ± 0.00
Candida tropicalis	KXF5	2.38 ± 0.21	0.08 ± 0.00
Candida novaki	TXF21	3.04 ± 0.20	0.12 ± 0.01
Candida novaki	TXF44	3.13 ± 0.11	0.12 ± 0.01
Candida sp.	TXF2–4	4.01 ± 0.05	0.08 ± 0.00
Candida quercuum	IXF5	3.71 ± 0.13	0.08 ± 0.00
Candida solani	IXF6	3.55 ± 0.17	0.18 ± 0.02
Candida solani	IXF7	3.72 ± 0.14	0.17 ± 0.02

One loop of yeast cells was inoculated in 5 ml of fermentation medium containing 5% xylose and incubated at 120 rpm and 30 °C for 48 h. Results are means ± SD for three independent experiments.

Table 2
Ethanol production activities from xylose of wild-type and mutant strains.

Strain		Xylose (%(w/v))	Ethanol (%(w/v))	Yield (CS*) (%)
Blank		4.48 ± 0.12	–	–
P. stipitis	NBRC1687	0.77 ± 0.28	1.64 ± 0.11	74.9 ± 0.2
	PXF4	0.59 ± 0.33	1.74 ± 0.13**	76.0 ± 0.5
	PXF36	0.54 ± 0.20	1.73 ± 0.11**	74.6 ± 1.0
	PXF58	0.17 ± 0.12	1.94 ± 0.07**	77.0 ± 0.8

Pre-culture (2.5 mL) was inoculated in 47.5 ml of fermentation medium containing 5% xylose and shaken at 120 rpm and 30 °C for 48 h. Results are means ± SD for three independent experiments.
* CS: consumed sugar.
** Significantly different from NBRC1687 ($p < 0.01$).

3.2. Fermentation characterization of *P. stipitis* PXF58 on sole sugar sources

PXF58 produced ethanol from xylose (Fig. 1A), glucose (Fig. 1B), and fructose (Fig. 1C) more rapidly and at a higher yields than NBRC1687 (Table 3). PFX58 produced around 4.2% ethanol from each sugar while the parental strain only produced around 3.0%. The ethanol yields of PXF58 were about 1.4 times higher than those of NBRC1687. Both NBRC1687 and PXF58 produced little ethanol (less than 0.1%) from sucrose and arabinose.

The catalytic pathway for converting xylose into xylulose 5-phosphate and the metabolic pathways of the pentose phosphate pathway (PPP) and glycolysis are well known as the *P. stipitis* mechanism for converting xylose into ethanol (Matsushika and Sawayama, 2008). However, the simultaneous increase in ethanol production on xylose, glucose and fructose suggests that the mutation(s) in PXF58 was/were not located in a xylose-specific gene(s), but in a gene or genes involved in sugar metabolism in general such as, perhaps, pyruvate decarboxylase or alcohol dehydrogenase. The mutation(s) did not result in morphological or growth differences on YPD medium.

Slininger et al. (1985) reported that *P. stipitis* NRRL Y-7124 (NBRC1687) produced 5.2% ethanol from 15% xylose in 150 h. When YX medium containing 1% yeast extract and 15% xylose was used, PXF58 produced 5.4% ethanol in 72 h (data not shown). Although the comparison of ethanol production rates in different fermenting media is difficult, PXF58 produced the same amount of ethanol in half the time, suggesting a more efficient ethanol conversion from xylose. The ethanol productivity could have been affected by substrate variation. Thus, further experiments using lignocellulosic biomass (such as wood and rice straw) are needed.

3.3. Fermentation characterization of *P. stipitis* PFX58 on mixed sugars

The fermentations of *P. stipitis* NBRC1687 and PXF58 with a mixed sugars yielded identical results (Fig. 2). Although PXF58 rapidly produced ethanol from glucose, xylose was not consumed. It has been reported that glucose represses xylose fermentation in a phenomenon known as "glucose repression" (Panchal et al., 1988). However, it appears that repression of xylose fermentation by glucose was not responsible since, even after the glucose was consumed, no ethanol was produced from xylose; rather, the ethanol produced from glucose decreased cell viability of PXF58 and NBRC1687 to 0.2% and 0.5%, respectively. Although PXF58 grew aerobically in YNBX medium containing 7% ethanol, this tolerance might not be the same under microaerobic conditions.

It has been reported that an adaptation technique improved the inhibitor tolerance of *P. stipitis* (Nigam, 2001; Chiung-Fang et al., 2009). Therefore, we attempted to improve the ethanol tolerance of PXF58 using an adaptation technique. Cultivation in the presence of increasing ethanol concentrations (5–7%) in microaerobic conditions was done for 20 cycles, and this process resulted in the isolation of a variant, PET41, that was able to grown in YNBX medium containing 10% ethanol in aerobic conditions (data not shown).

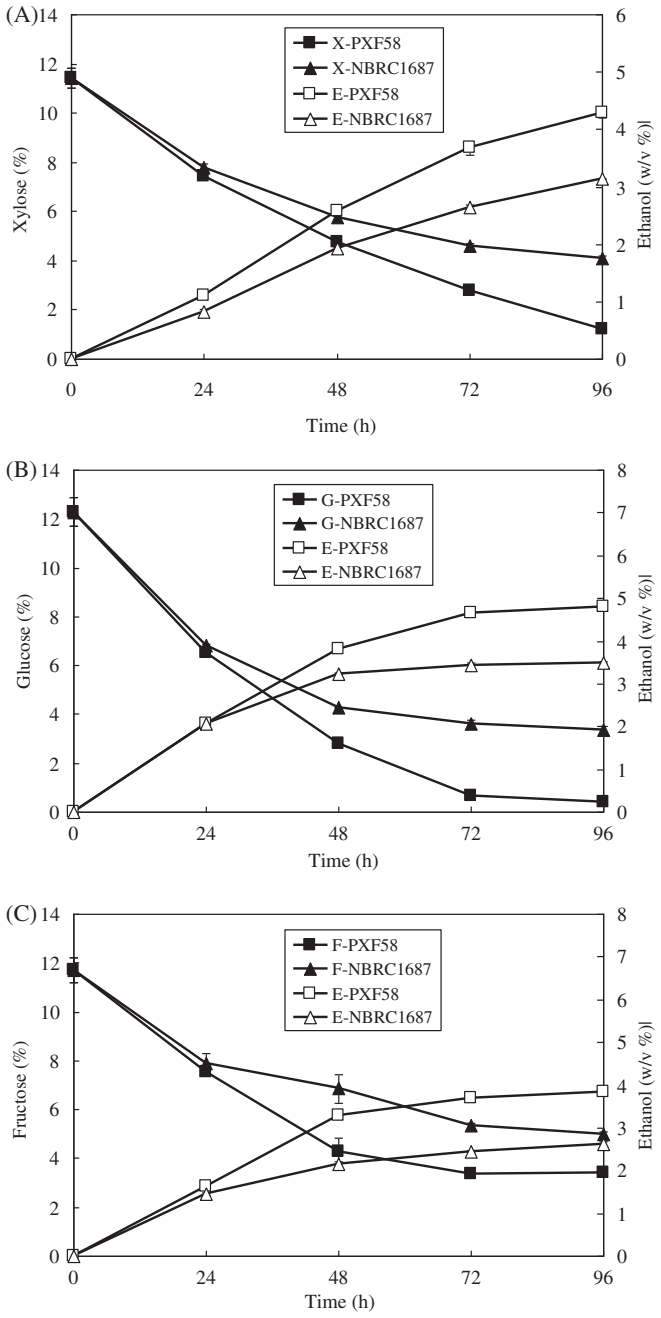

Fig. 1. Time course of ethanol production from the medium containing 12% xylose (A), 15% glucose (B), and 15% fructose (C) by *P. stipitis* PXF58 (squares) and *P. stipitis* NBRC1687 (triangles). The left and right Y-axes represent sugar (filled symbols) and ethanol concentrations (empty symbols), respectively. Yeast strains were cultivated in 100-ml flasks with 45 ml of fermentation medium (10% of inoculum) shaken at 120 rpm and 30 °C. Results shown are means of three independent experiments.

3.4. Fermentation performances in two-phase fermentations

PET41 produced ethanol from xylose and was still able to convert the added glucose into ethanol to produce a total of 4.4% ethanol after 96 h (Fig. 3C), whereas NBRC1687 (Fig. 3A) and PXF58

Table 3
The fermentation performances of *P. stipitis* PXF58 and *P. stipitis* NBRC1687 using fermentation media of three sugar types at 30 °C.

Parameter	*P. stipitis* PXF58			*P. stipitis* NBRC1687		
	Xylose	Glucose	Fructose	Xylose	Glucose	Fructose
Total sugar (%(w/v))	11.4 ± 0.4	12.3 ± 0.6	11.7 ± 0.5	11.4 ± 0.4	12.3 ± 0.6	11.7 ± 0.5
Residual sugar (%(w/v))	1.2 ± 0.1*	0.7 ± 0.2*	3.4 ± 0.1*	4.1 ± 0.1	3.6 ± 0.1	5.0 ± 0.1
Ethanol (%(w/v))	4.3 ± 0.1*	4.7 ± 0.1*	3.7 ± 0.1*	3.1 ± 0.1	3.4 ± 0.2	2.6 ± 0.1
Yield (Total sugar) (%)	68.1 ± 0.9*	74.2 ± 0.8*	61.8 ± 0.6*	49.8 ± 0.9	54.7 ± 0.8	43.6 ± 0.6
Yield (Consumed sugar) (%)	76.0 ± 1.0	78.6 ± 0.9	87.0 ± 0.7	77.4 ± 1.1	77.5 ± 0.9	75.9 ± 0.7
Fermentation time (h)	96	72	96	96	72	96
Maximum productivity (g/L/h)	0.6 ± 0.0	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.9 ± 0.1	0.6 ± 0.0

Results are means ± SD for three independent experiments.
* Significantly different from NBRC1687 ($p < 0.01$).

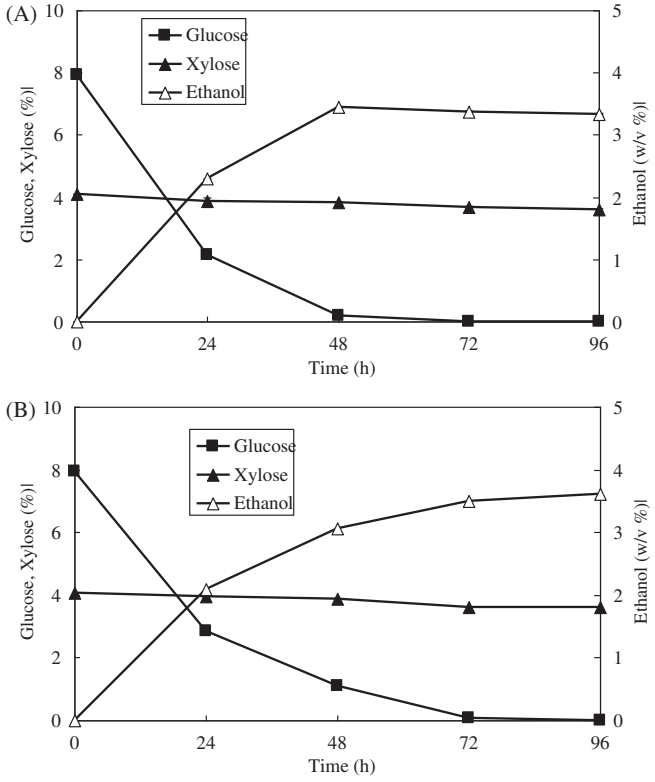

Fig. 2. Time course of ethanol production from mixed-sugar fermentation medium using *P. stipitis* NBRC1687 (A) and *P. stipitis* PXF58 (B). The left Y-axis represents glucose (filled squares) and xylose concentrations (filled triangles). The right Y-axis represents the ethanol concentration (empty triangles). Yeast strains were cultivated in 100-ml flasks with 45 ml of fermentation medium (10% of inoculum) shaken at 120 rpm and 30 °C. Results shown are means of three independent experiments.

(Fig. 3B) produced 3.8% and 4.0% ethanol, respectively. This increase in performance was likely due to increased ethanol tolerance.

Li et al. (2010) reported that 2.2% ethanol was produced from aqueous ammonia pretreated corn stover with TPSSF using recombinant *E. coli* KO11 and *S. cerevisiae* D5A. Although the comparison of ethanol productivities between from lignocellulosic hydrolysate and YNB medium was difficult, a non-recombinant strain PET41 would be more easily utilized for industrial application than recombinant strain.

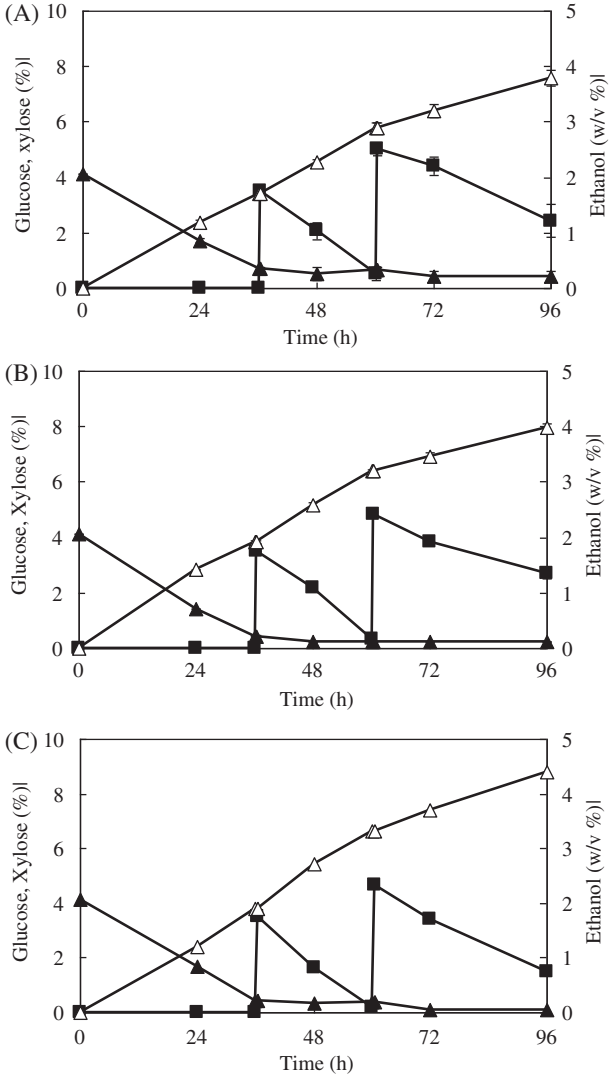

Fig. 3. Time course of ethanol production by two-phase fermentation using *P. stipitis* NBRC1687 (A), *P. stipitis* PXF58 (B), and *P. stipitis* PET41 (C). The left Y-axis represents glucose (filled squares) and xylose concentrations (filled triangles). The right Y-axis represents the ethanol concentration (open triangles). Yeast strains were cultivated in 100-ml flasks with 40 ml of fermentation medium (10% of inoculum) containing 4.5% xylose shaken at 120 rpm and 30 °C. Glucose solutions were added to the culture medium at 36 and 60 h. Results shown are means of three independent experiments.

4. Conclusions

Efficient ethanol production from xylose is crucial for bioethanol production from lignocellulosic biomass. We attempted to enhance the ethanol productivity of the xylose-fermenting yeast *P. stipites*. Using UV-mutagenesis and a strategy of repeated exposure to increasing ethanol concentrations, a mutant of *P. stipitis*

NBRC1687 was isolated that produced more ethanol from xylose than the wild-type and that appears suitable for use in TPSSF of lignocellulosic biomass. The nature of the mutations in this strain still remains to be elucidated.

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