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Deletion of the *PHO13* gene in *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and formic acids, and furfural

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ABSTRACT

For efficient bioethanol production from lignocellulosic biomass by *Saccharomyces cerevisiae*, it is necessary to improve cellular tolerance to toxic compounds released during the pretreatment of biomass. The gene encoding *p*-nitrophenylphosphatase, *PHO13*, was disrupted in a recombinant xylose-fermenting *S. cerevisiae* strain, which improved ethanol production from xylose in the presence of three major inhibitors, acetic and formic acids, and furfural. In medium supplemented with 30 mM acetic acid, the ethanol yield obtained by the Δ*PHO13* mutant was 0.45 g-ethanol/g-xylose. Notably, the specific ethanol productivity of the mutant in the presence of 90 mM furfural was fourfold higher than that of the control strain. The *PHO13*-disrupted strain produced ethanol from rice straw hydrolysate obtained by liquid hot-water pretreatment with a greater than fourfold higher xylose consumption rate than the control. Together, our findings demonstrate that *PHO13* deletion is a simple, but effective, approach for improving cellulosic bioethanol production by *S. cerevisiae*.

1. Introduction

Ethanol production from plant biomass has received considerable recent attention because of the expectation that bioethanol will alleviate demands for petroleum-based fuels. Lignocellulosic materials, such as wood and agricultural residues, represent a sustainable energy resource and do not compete with food and animal feed production. The hydrolysis of lignocellulosic biomass, consisting of cellulose, hemicellulose, and lignin, liberates sugars, primarily glucose and xylose, which are subsequently fermented by microorganisms to produce ethanol (Saka, 1991). The yeast *Saccharomyces cerevisiae* is a promising candidate for industrial bioethanol production due to its robustness and high ethanol productivity. However, the economic viability of using lignocellulosic biomass for ethanol production is highly dependent upon the bioconversion efficiency of hemicelluloses, which predominantly consist of xylose (Galbe et al., 2007), which cannot be naturally fermented by *S. cerevisiae* (Hahn-Hägerdal et al., 2001).

In the last few decades, genetic engineering approaches have enabled *S. cerevisiae* to utilize xylose through the expression of heterologous genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces* (*Pichia*) *stipitis*, together with the endogenous gene for xylulokinase (XK) (Chu and Lee, 2007; Eliasson et al., 2000). In this engineered pathway, XR first reduces xylose to xylitol, which is then oxidized to xylulose by the action of XDH. Xylulose is subsequently phosphorylated by XK to xylulose 5-phosphate, which is metabolized through the pentose phosphate pathway and glycolysis to form ethanol. However, the rapid and efficient fermentation of hemicellulosic hydrolysates by this engineered *S. cerevisiae* strain is still limited by the toxic compounds generated during the biomass pretreatment process (reviewed in Palmqvist and Hahn-Hägerdal, 2000a,b). These inhibiting compounds negatively affect cell growth, metabolism, and ethanol yield (van Maris et al., 2006), and are categorized in the following three main groups based on their origin: weak acids, furan derivatives, and phenolic compounds (reviewed in Palmqvist and Hahn-Hägerdal, 2000a). In xylose-fermenting recombinant *S. cerevisiae* strains, xylose utilization was more severely affected by the addition of inhibitors such as acetic acid, formic acid, and furfural than was glucose utilization (Bellissimi et al., 2009; Hasunuma et al., 2011a). Therefore, to overcome the toxicity of these compounds to *S. cerevisiae*, the ability to efficiently utilize xylose is an essential requirement for the effective production of bioethanol from lignocellulosic hydrolysates.

A recent study has suggested that deleting the gene encoding *p*-nitrophenylphosphatase, *PHO13*, in recombinant *S. cerevisiae* strains improves growth and ethanol production from xylose under aerobic and microaerobic conditions (van Vleet et al., 2008). However, no research has investigated the effect of *PHO13*

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deletion on xylose fermentation in the presence of fermentation inhibitors. Here, a recombinant xylose-fermenting *S. cerevisiae* strain containing a disrupted *PHO13* gene was constructed to evaluate ethanol productivity in the presence of the fermentation inhibitors such as acetic and formic acids, and furfural.

2. Methods

2.1. Strains

Escherichia coli strain NovaBlue (Novagen Inc., Madison, WI) was used as a host for recombinant DNA manipulation and was routinely cultured using LB medium supplemented with 100 mg/L ampicillin at 37 °C. *S. cerevisiae* strains BY4741 and BY4741/Δ*PHO13* were purchased from Invitrogen (Tokyo, Japan). The growth media and culture conditions for these strains have been described previously (Hasunuma et al., 2011a).

2.2. Yeast transformation

Plasmid pIUX1X2XK (Katahira et al., 2006) was digested with *Eco*RV and transformed into strains BY4741 and BY4741/Δ*PHO13* to yield BY4741X and BY4741X/Δ*PHO13*, respectively. Transformation was performed by the lithium-acetate method, as previously described (Katahira et al., 2006). Transformants were selected on synthetic dextrose (SD) medium (6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI] and 20 g/L glucose) supplemented with appropriate amino acids and nucleotides.

2.3. Yeast growth assay under aerobic conditions

After pre-incubation in SD medium for 24 h, yeast cells were collected by centrifugation at 1000*g* for 5 min at 4 °C and inoculated into yeast extract/peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) containing 20 g/L xylose. The initial cell concentration was adjusted to an optical density at 600 nm (OD600) of 0.05. Aerobic batch cultivations were performed by shaking at 70 rpm at 30 °C for 120 h using a Bio-photorecorder (TVS062CA; Advantec Toyo, Tokyo, Japan).

2.4. Batch fermentation under oxygen-limited conditions

After pre-incubation in SD medium for 24 h, yeast cells were cultured under aerobic conditions for 48 h at 30 °C in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). Cells were collected by centrifugation at 1000*g* for 5 min at 4 °C, washed twice with distilled water, and then inoculated into YPX fermentation medium (10 g/L yeast extract, 20 g/L peptone, and 80 g/L xylose) containing specified concentrations of fermentation inhibitors (acetic acid, formic acid, and furfural). All fermentations were performed at 30 °C with an agitation speed of 500 rpm in 100-mL closed bottles equipped with a bubbling CO_2 outlet and a stirrer bar. Agitation speed was maintained with a magnetic stirrer, VARIOMAG Telesystem (Thermo Scientific, Tokyo, Japan). In taking fermentation medium, the lid of the fermentation bottle was opened. The dissolved oxygen concentration was 10.8% (v/v) during the sampling, which was immediately decreased to less than 0.18% (v/v) after restart of the fermentation. The dissolved oxygen concentration was measured with Fibox 3 Trace (PreSens, Regensburg, Germany). The initial cell concentration was adjusted to 50 g wet cells/L. Wet cell weight was determined by weighing cell pellets that were harvested by centrifugation from the 48-h YPD cultures.

2.5. Fermentation of lignocellulosic hydrolysate

A lignocellulosic hydrolysate prepared by liquid hot water treatment was obtained as described previously (Sanda et al., 2011). The hydrolysate was composed of sugars (11.99 g/L glucose, 9.66 g/L xylose, 2.58 g/L arabinose, 0.89 g/L galactose, and 4.72 g/L fructose), 27.11 mM acetic acid, 20.06 mM formic acid, 7.77 mM furfural, 0.46 mM 5-HMF, 0.56 mM vanillin, and 0.37 mM syringaldehyde, and was used as a fermentation medium after supplementation with 10 g/L yeast extract and 20 g/L peptone. The fermentation was performed as described above.

2.6. Measurement of fermentation products

Cell concentration was determined by measuring the OD600. The concentrations of ethanol, glycerol, xylitol, and xylose in the fermentation medium were determined as described previously (Hasunuma et al., 2011a). Acetate and formate were measured using an HPLC system (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (7.8 × 300 mm; Bio-Rad, Hercules, CA) according to the method of Almeida et al. (2009).

2.7. Gene expression profiling and analysis

Total RNA was obtained after 9 h fermentation in the absence of fermentation inhibitors by using the Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA) following the manufacturer's protocol. cDNA was generated by reverse transcription, labeled with Cy3, and hybridized to *S. cerevisiae* 4 × 44k microarrays. Array scans and data analyses were performed with the Agilent DNA Microarray Scanner and Genespring GX software, respectively (Agilent Technologies). Gene expression levels were normalized per chip.

3. Results and discussion

3.1. Growth under aerobic conditions

Xyl1 and *Xyl2* encoding XR and XDH, respectively, from *S. stipitis*, and *Xks1* encoding XK from *S. cerevisiae* were integrated into the genome of the *PHO13*-deleted *S. cerevisiae* strain to yield a recombinant xylose-fermenting Δ*PHO13* strain, BY4741X/Δ*PHO13*. As a control strain, *Xyl1*, *Xyl2*, and *Xks1* were integrated into the genome of strain BY4741 to give BY4741X. The integration of the three genes into the chromosomes of the respective host strains was confirmed by genomic PCR (data not shown).

The effects of *PHO13* deletion on the cell growth in YP medium containing 20 g/L xylose as a sole carbon source were investigated under aerobic conditions (Fig. 1). The initial OD600 of the culture was 0.05. Although no significant differences in the length of the log phase were detected between the control and Δ*PHO13* mutant strains, the lag phase of the Δ*PHO13* mutant ended approximately 50-h sooner than that of the control. Previously, the deletion of *PHO13* enhanced the specific growth rate of a xylose-fermenting CEN.PK strain (van Vleet et al., 2008). These results indicate that the effect of *PHO13* deletion on cell growth on xylose is observed even with differing genetic background.

3.2. Xylose fermentation under oxygen-limited conditions

The effect of *PHO13* deletion on xylose fermentation under oxygen-limited conditions was evaluated. The fermentation was performed in YP medium containing 80 g/L xylose as the sole carbon source at 30 °C after first cultivating cells aerobically in YPD medium. In both control strain and Δ*PHO13* mutant, pH value of

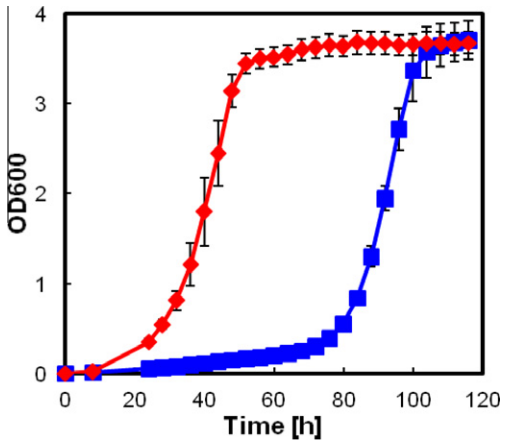

Fig. 1. Aerobic batch cultivations of xylose-fermenting recombinant *S. cerevisiae* strains BY4741X (squares) and BY4741X/Δ*PHO13* (diamonds). The OD600 values of cultures represent the averages of three independent experiments ± SEM.

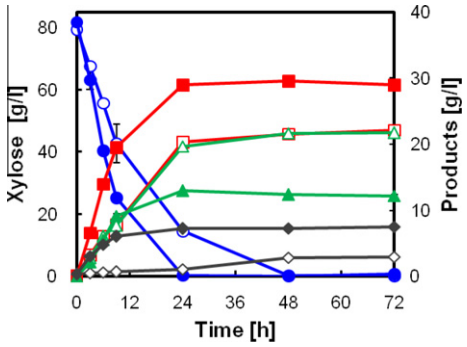

Fig. 2. Xylose fermentation by strains BY4741X (open symbols) and BY4741X/Δ*PHO13* (closed symbols) under oxygen-limited conditions. Xylose (circles), ethanol (squares), xylitol (triangles) and glycerol (diamonds) concentrations were measured over the 72-h fermentation period. Values represent the averages of three independent experiments ± SEM.

fermentation medium decreased gradually after starting fermentation to reach 4.7 after 72-h fermentation. As shown in Fig. 2, the Δ*PHO13* mutant produced 33.9 g/L after 72 h, whereas the control strain only generated 25.4 g/L ethanol during the same period. The mutant strain also exhibited a twofold higher volumetric xylose consumption rate and a 2.6-fold higher volumetric ethanol productivity compared to the control strain (Table 1). The specific rate of xylose consumption and ethanol production by the Δ*PHO13* mutant were 0.44 g-xylose/g-cells/h and 0.15 g-ethanol/g-cells/h, respectively, whereas those of the control strain were 0.16 g-xylose/g-cells/h and 0.05 g-ethanol/g-cells/h (Table 1). These results indicate that the deletion of *PHO13* in the recombinant xylose-fermenting *S. cerevisiae* strain improves ethanol production ability from xylose under oxygen-limited conditions. Our findings are consistent with a previous report that a xylose-fermenting recombinant strain lacking functional *PHO13* produced approximately 1.0 g/L ethanol from 20 g/L xylose as the sole carbon source in a minimal medium under low oxygen conditions, while the control strain produced almost no ethanol (van Vleet et al., 2008). Here, the Δ*PHO13* mutant produced higher ethanol (29 g/L) by utilizing higher xylose (80 g/L) with 71% of the theoretical yield after only 24 h fermentation than the previous report (van Vleet et al., 2008). This study revealed that the positive effect of deleting *PHO13* on ethanol production was observed at highly-concentrated xylose fermentation. van Vleet et al. (2008) also observed a 10-fold decrease in glycerol accumulation in culture supernatants compared to controls that may have been a result of higher levels of glycerol kinase, which converts glycerol to glycerol-3-phosphate. In contrast, the Δ*PHO13* mutant accumulated much higher glycerol concentrations than that of control strain. The glycerol production is closely related to the balance of intracellular NAD^+/NADH ratio (Bruinenberg at al., 1983). The opposite effect on glycerol production might be attributed to the difference in the redox balance between strains.

3.3. Effect of acetic acid on the PHO13-deleted recombinant strain

The effect of acetic acid (30 and 60 mM) on xylose fermentation by the Δ*PHO13* mutant was next investigated. The fermentation was performed as described above, with the exception of the addition of acetic acid to the medium. During the fermentation, pH value was almost constant. The pH value observed after 72-h fermentation was similar between with and without acetate addition. The Δ*PHO13* mutant showed higher ethanol production and xylose consumption rates than the control strain at both examined acetic acid concentrations (Fig. 3). The specific xylose consumption rates of Δ*PHO13* mutant were 0.25 and 0.09 g-xylose/g-cells/h at 30 and 60 mM acetate, respectively, while those of the control strain were 0.10 and 0.05 g-xylose/g-cells/h (Table 1). The specific ethanol productivities of Δ*PHO13* mutant were 0.08 and 0.04 g-ethanol/g-cells/h at acetate concentrations of 30 and 60 mM, respectively, while those of the control strain were 0.04 and 0.01 g-ethanol/g-cells/h (Table 1). Furthermore, the deletion of *PHO13* led to higher ethanol production and yield in the presence of 30 and 60 mM acetate, although xylose consumption of the Δ*PHO13* mutant was similar to the control strain in the presence of 60 mM acetate after 72 h fermentation. The maximum ethanol yield from the initial xylose present in the fermentation medium containing 30 and 60 mM acetic acid was 0.45 and 0.35 g-ethanol/g-xylose, respectively. Acetic acid concentration slightly increased during the fermentation (Fig. S1).

Previously, improvement of ethanol production in the presence of 30 and 60 mM acetic acid was achieved by the overexpression of the *S. cerevisiae* transaldolase (*TAL1*) gene, which is involved in the pentose phosphate pathway (Hasunuma et al., 2011a). Notably, although the *TAL1*-expressing strain showed the highest ethanol productivity so far, the ethanol yield of the Δ*PHO13* mutant achieved here was slightly higher than that of the *TAL1*-expressing strain.

The addition of 30 mM acetate to the fermentation medium increased ethanol production by both the control strain and Δ*PHO13* mutant, a result that was likely based on the uncoupling theory (Verduyn et al., 1992). The theory states that a drop in intracellular pH resulting from the inflow of weak acid is neutralized by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis (Verduyn et al., 1992). Thus, additional ATP must be generated to maintain the intracellular pH, and under anaerobic conditions, ATP generation is achieved by ethanol production (Viegas and Sá-Correia, 1991).

3.4. Effect of formic acid on the PHO13-deleted recombinant strain

Xylose fermentation was also performed in the presence of 15 and 30 mM formic acid. The pH value was almost constant during fermentation. According to previous reports (Hasunuma et al., 2011a; Larsson et al., 2001), formic acid has a greater inhibitory effect on the yield of ethanol from xylose fermentation than that of acetic acid. The increased toxicity of formic acid appears to be associated with its smaller molecule size, which may facilitate diffusion through the plasma membrane, possibly leading to higher anion toxicity (reviewed in Almeida et al., 2007). Here, in medium supplemented with 30 mM formic acid, the control strain produced

Table 1
Yields and productivities of ethanol from xylose in the presence and absence of fermentation inhibitors under oxygen-limited conditions[a].

Strain	Inhibitor	Conc. (mM)	Initial pH of fermentation	Q_{max}[b]	$Y^{max}_{E/TS}$[c]	q_{Smax}[d]	q_{Emax}[e]	$Y_{x/TS}$[f]
BY4741X	No inhibitor		6.1	0.826 ± 0.002	0.279 ± 0.002	0.155 ± 0.004	0.049 ± 0.002	0.653 ± 0.004
	Acetic acid	30	4.6	0.607 ± 0.001	0.331 ± 0.003	0.103 ± 0.006	0.036 ± 0.004	0.641 ± 0.002
		60	4.4	0.201 ± 0.008	0.274 ± 0.004	0.054 ± 0.009	0.013 ± 0.005	0.936 ± 0.008
	Formic acid	15	4.7	0.482 ± 0.005	0.360 ± 0.001	0.100 ± 0.002	0.033 ± 0.002	0.780 ± 0.005
		30	4.1	0.059 ± 0.010	0.286 ± 0.002	0.014 ± 0.005	0.004 ± 0.009	5.503 ± 0.006
	Furfural	60	5.9	0.320 ± 0.012	0.292 ± 0.010	0.025 ± 0.001	0.021 ± 0.003	0.742 ± 0.009
		90	5.8	0.101 ± 0.002	0.281 ± 0.009	0.022 ± 0.002	0.008 ± 0.004	1.579 ± 0.007
BY4741X/Δ*PHO13*	No inhibitor		6.1	2.184 ± 0.008	0.357 ± 0.012	0.435 ± 0.010	0.148 ± 0.011	0.531 ± 0.009
	Acetic acid	30	4.7	1.052 ± 0.011	0.451 ± 0.010	0.254 ± 0.009	0.080 ± 0.007	0.493 ± 0.007
		60	4.4	0.534 ± 0.013	0.349 ± 0.009	0.086 ± 0.006	0.039 ± 0.010	0.652 ± 0.004
	Formic acid	15	4.8	1.611 ± 0.019	0.365 ± 0.015	0.339 ± 0.011	0.114 ± 0.012	0.504 ± 0.001
		30	4.1	0.297 ± 0.009	0.307 ± 0.011	0.077 ± 0.013	0.069 ± 0.011	1.028 ± 0.014
	Furfural	60	6.0	1.220 ± 0.016	0.414 ± 0.003	0.200 ± 0.015	0.077 ± 0.008	0.559 ± 0.012
		90	5.8	0.942 ± 0.014	0.286 ± 0.012	0.170 ± 0.018	0.066 ± 0.013	0.504 ± 0.010

[bde] Maximum volumetric productivity, maximum specific xylose consumption rate, and maximum specific productivity were calculated from the change in ethanol and xylose with respect to time from 3 to 9 h.
[a] Values are averages of three independent experiments ± SEM. N.D., not detected.
[b] Maximum volumetric productivity (g/L/h).
[c] Maximum ethanol yield on total sugar (g-ethanol/g-xylose).
[d] Maximum specific xylose consumption rate (g-xylose/g-cells/h).
[e] Maximum specific productivity (g-ethanol/g-cells/h).
[f] Biomass yield on total sugar (g-cells/g-xylose).

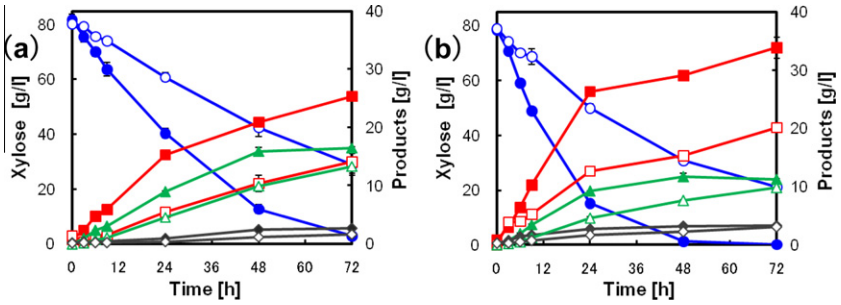

Fig. 3. Effect of acetic acid on xylose fermentation by strains BY4741X (a) and BY4741X/Δ*PHO13* (b). Xylose (circles), ethanol (squares), xylitol (triangles) and glycerol (diamonds) concentrations in the presence of 30 mM (closed symbols) and 60 mM acetate (open symbols) were measured over the 72-h fermentation period. Values represent the averages of three independent experiments ± SEM.

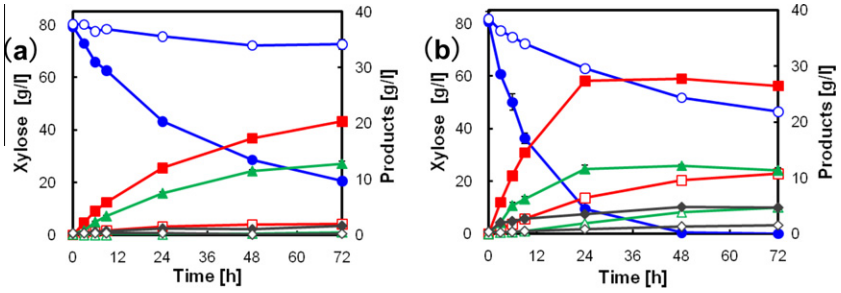

Fig. 4. Effect of formic acid on xylose fermentation by strains BY4741X (a) and BY4741X/Δ*PHO13* (b). Xylose (circles), ethanol (squares), xylitol (triangles) and glycerol (diamonds) concentrations in the presence of 15 mM (closed symbols) and 30 mM formate (open symbols) were measured over the 72-h fermentation period. Values represent the averages of three independent experiments ± SEM.

only 2.0 g/L ethanol from 80 g/L xylose, whereas the Δ*PHO13* mutant was able to generate 10.8 g/L ethanol (Fig. 4). In the presence of 15 mM formic acid, the Δ*PHO13* mutant completely consumed xylose after 48 h fermentation to produce 26.5 g/L ethanol, while greater than 20 g/L xylose remained in the medium of the control strain after 72 h fermentation (Fig. 4). The specific xylose consumption rates of the Δ*PHO13* mutant were 0.34 and 0.08 g-xylose/g-cells/h in the presence of 15 and 30 mM formic acid, respectively, while those of the control strain were 0.10 and 0.01 g-xylose/g-cells/h, respectively (Table 1).

Previously, the expression of *FDH1*, encoding formate dehydrogenase, by a metabolically engineered *S. cerevisiae* strain reduced formic acid levels in the medium and led to increased ethanol production from xylose (Hasunuma et al., 2011). However, fermentation by the Δ*PHO13* mutant slightly increased formic acid concentration in the medium, regardless of the initial formic acid

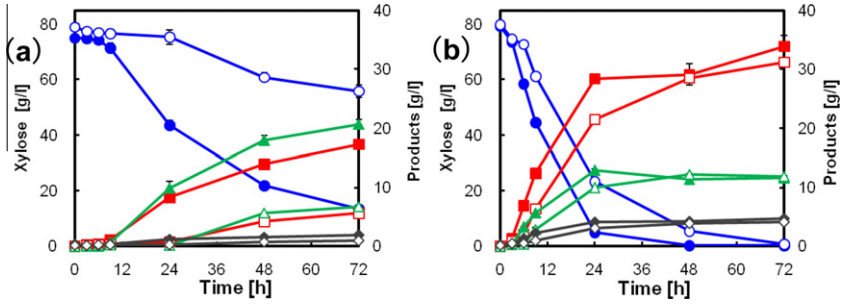

Fig. 5. Effect of furfural on xylose fermentation by BY4741X (a) and BY4741X/Δ*PHO13* (b). Xylose (circles), ethanol (squares), xylitol (triangles) and glycerol (diamonds) concentrations in the presence of 60 mM (closed symbols) and 90 mM furfural (open symbols) were measured over the 72-h fermentation period. Values represent the averages of three independent experiments ± SEM.

level (Fig. S2). The improvement of ethanol production might be explained by the uncoupling theory as described in the case of acetate addition, while further studies are required for the elucidation of the inhibitory mechanism. Recently, Sanda et al. (2011) reported that a recombinant xylose-fermenting *S. cerevisiae* strain expressing both transaldolase (*TAL1*) and formate dehydrogenase (*FDH1*) genes also gave higher ethanol yields when exposed to formic acid. Under 20 mM formate conditions, the ethanol production rate of a diploid strain expressing two sets of *TAL1* and *FDH1* was approximately 1.17 g/L/h, whereas that of a Δ*PHO13* mutant haploid strain was 1.61 g/L/h in the presence of 15 mM formate.

3.5. Effect of furfural on the PHO13-deleted recombinant strain

Furan aldehydes such as furfural and 5-hydroxymethylfurfural reduce the growth and ethanol production rates of yeast cells (Boyer et al., 1992). The addition of furan derivatives into the fermentation medium did not affect pH value of the fermentation medium. Thus, the xylose fermentations were performed with the *PHO13*-deleted strain in the presence of 60 and 90 mM furfural. As shown in Fig. 5, the control strain was highly adversely affected by furfural toxicity at both examined concentrations and only utilized 30% of the available xylose. In contrast, the Δ*PHO13* mutant was able to overcome the toxicity of 60 and 90 mM furfural and produce ethanol at higher yields than the control strain (Fig. 5). The specific xylose consumption rates of the Δ*PHO13* mutant were 0.20 and 0.17 g-xylose/g-cells/h at furfural concentrations of 60 and 90 mM, respectively, while those of the control strain were 0.03 and 0.02 g-xylose/g-cells/h (Table 1). The specific ethanol productivities of Δ*PHO13* mutant were 0.08 and 0.07 g-ethanol/g-cells/h at furfural concentrations of 60 and 90 mM, respectively, whereas those of the control strain were only 0.02 and 0.01 g-ethanol/g-cells/h.

In this study, xylose fermentation by a recombinant xylose-fermenting strain of *S. cerevisiae* lacking *PHO13* was examined in the presence of three major fermentation inhibitors derived from lignocellulose. All of the inhibitors have been reported to inhibit growth and retard fermentation by their toxicity for cellular function and components, such as transport systems, mitochondria, and vacuole membranes (reviewed in Palmqvist and Hahn-Hägerdal, 2000a), although the mechanisms of inhibition by each compound are not completely understood. Interestingly, the deletion of *PHO13* improved ethanol production from xylose in the presence of all three examined fermentation inhibitors.

PHO13 protein has been found to possess phosphoprotein phosphatase activity toward histone phosphorylated at tyrosine residues (Kaneko et al., 1989). Although the function of PHO13 protein remains unclear, deletion of the *PHO13* gene results in conditions within the intracellular environment that is conducive for xylose fermentation. In fact, global gene expression analysis using a DNA microarray demonstrated that the expression of genes involved in the pentose phosphate pathway and glycolysis, including *ZWF1*, *SOL3*, and ADH1, was increased by deletion of the *PHO13* gene (Table S1). In contrast, the expression levels of respiratory chain-related genes, such as *COX2* and *CYC1*, and mitochondrial proton-transporting ATP synthase genes (*ATP1* and *ATP19*) in the *ΔPHO13* mutant were lower than those in the control strain. Recently, a few studies have suggested that xylose fermentation ability is related to mitochondrial function because the redox cofactors NADH and NADPH are involved in the catabolism and biosynthesis of metabolites during growth on xylose (Mo et al., 2009; Salusjarvi et al., 2003; Shi et al., 2002). Therefore, deleting the *PHO13* gene from *S. cerevisiae* possibly conferred a positive effect on not only xylose assimilation, but also on diverse cellular metabolic machinery.

3.6. Fermentation of lignocellulosic hydrolysates

Rice straw hydrolysates obtained by liquid hot water treatment and subsequent enzymatic treatment were used for fermentation. The hydrolysates were composed of 11.99 g/L glucose, 9.66 g/L xylose, 4.72 g/L fructose, 2.58 g/L arabinose, and 0.89 g/L galactose, and also contained several fermentation inhibitors, including 27.11 mM acetic acid, 20.06 mM formic acid, 7.77 mM furfural, 0.46 mM 5-HMF, 0.56 mM vanillin, and 0.37 mM syringaldehyde. Xylose consumption rates in fermentations conducted with rice straw hydrolysates by the Δ*PHO13* mutant and control strain were 0.50 and 0.11 g-xylose/l/h, respectively (Fig. 6). Slight amount of xylitol was produced during the fermentation. These results confirm that deletion of the *PHO13* gene improved ethanol production from lignocellulosic hydrolysate containing multiple fermentation inhibitors.

In general, the use of common laboratory haploid strains has low feasibility for industrial bioethanol production, because these strains have lower tolerance to temperature fluctuations, ethanol, and fermentation inhibitors than industrial diploid and polyploid strains (Garay et al., 2004). In this study, a haploid strain with a deleted *PHO13* gene was tested for xylose fermentation in the presence of several inhibitors and showed a higher ability to produce ethanol compared to previously reported diploid strains (Sanda et al., 2011). Therefore, the use of diploid and polyploid strains lacking a functional *PHO13* gene is a promising approach for improving ethanol production from lignocellulosic hydrolysates. Furthermore, as gene disruption simply represents the loss of a gene sequence, the genetic background of recombinant strains obtained would be more robust than strains over-expressing specific target genes (Akada, 2002). Further studies using industrial strains as hosts for molecular breeding would provide low-cost ethanol production from lignocellulosic hydrolysates.

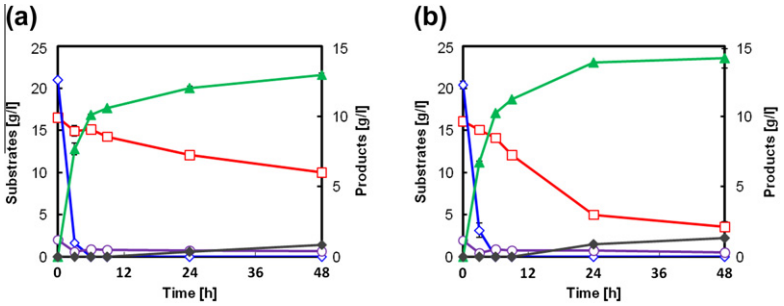

Fig. 6. Batch fermentation of lignocellulosic hydrolysate by BY4741X (a) and BY4741X/Δ*PHO13* (b). The concentrations of glucose (open diamonds), fructose (open circles), xylose (open squares), ethanol (closed triangles), and xylitol (closed diamonds) are shown. Values represent the averages of three independent experiments ± SEM.

4. Conclusions

Deletion of the *PHO13* gene in a xylose-fermenting recombinant *S. cerevisiae* strain promoted cell growth under aerobic conditions and increased ethanol production under oxygen-limited conditions. In the presence of major the fermentation inhibitors acetic and formic acids, and furfural, the *PHO13*-deleted strain exhibited improved ethanol production from xylose. Moreover, the Δ*PHO13* mutant showed enhanced ethanol production from rice straw hydrolysates. This study demonstrates that deletion of the *PHO13* gene is a simple, but powerful, strategy for efficient ethanol production from lignocellulosic biomass.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.01.161.

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