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ORIGINAL RESEARCH PAPER

Synergistic effects of *TAL1* over-expression and *PHO13* deletion on the weak acid inhibition of xylose fermentation by industrial *Saccharomyces cerevisiae* strain

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Abstract In the industrial production of bioethanol from lignocellulosic biomass, a strain of *Saccharomyces cerevisiae* that can ferment xylose in the presence of inhibitors is of utmost importance. The recombinant, industrial-flocculating *S. cerevisiae* strain NAPX37, which can ferment xylose, was used as the parent to delete the gene encoding *p*-nitrophenylphosphatase (*PHO13*) and overexpress the gene encoding transaldolase (*TAL1*) to evaluate the synergistic effects of these two genes on xylose fermentation in the presence of weak acid inhibitors, including formic, acetic, or levulinic acids. *TAL1* over-expression or *PHO13* deletion improved xylose fermentation as well as the tolerance of NAPX37 to all three weak acids. The simultaneous deletion of *PHO13* and the over-expression of *TAL1* had synergistic effects and improved ethanol production and reduction of xylitol accumulation in the absence and presence of weak acid inhibitors.

Keywords Bioethanol · *PHO13* gene · *Saccharomyces cerevisiae* · *TAL1* gene · Weak acid inhibitors · Xylose fermentation

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Introduction

There is interest in the application of renewable resources, such as lignocellulosic biomass, for the production of bioethanol, the cleanest liquid fuel alternative to fossil fuels. Lignocellulosic biomass, such as energy crops, wood, and agricultural residues, primarily consist of cellulose, hemicelluloses, and lignin (Matsushika et al. 2009a; Kim et al. 2013). Lignocellulosic biomass is the most abundant, renewable carbohydrate source for bioethanol production and does not compete with food supplies (Matsushika et al. 2009a; Ismail et al. 2013). Xylose is the second most abundant monosaccharide present in the hydrolysate of lignocellulosic biomass and should be fermented to ethanol to develop an economically feasible process of lignocellulosic bioethanol production (Van Vleet et al. 2008; Matsushika et al. 2012).

Saccharomyces cerevisiae, amongst numerous fermentative microorganisms, is an industrial producer of ethanol due to its physiology and tolerance to ethanol (Van Vleet et al. 2008). Unfortunately, *S. cerevisiae* cannot utilize pentoses, especially D-xylose, to produce ethanol. Furthermore, some inhibitors are inevitably present in the hydrolysate of lignocellulosic biomass and have negative effects on the growth and fermentation of yeast strains during bioethanol production. Therefore, extensive researches had been performed to engineer *S. cerevisiae* strains with the ability to metabolize xylose and tolerate inhibitors. Although significant progress has been made (Van Vleet and Jeffries 2009; Almeida et al. 2011; Jönsson et al. 2013), more research still needs to be conducted on the industrial production of cellulosic ethanol.

Although *S. cerevisiae* is unable to metabolize xylose, it has the metabolic pathway to convert xylulose, a keto-isomer of D-xylose, into ethanol. Xylulose can be phosphorylated to xylulose 5-phosphate by xylulokinase (XK) and degraded via the pentose phosphate pathway then channeled into glycolysis. Numerous studies have been conducted to link extracellular xylose with intracellular xylulose to further improve the efficiency of xylose fermentation (Matsushika et al. 2009a). One key advancement made in these genetic engineering studies was the heterologous expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces* (*Pichia*) *stipities,* along with over-expression of XK. In this pathway, XR reduces xylose to xylitol, which is then oxidized to xylulose by XDH (Fujitomi et al. 2012; Kato et al. 2012). However, the fermentation rates and ethanol yields from lignocellulosic hydrolysate by these engineered *S. cerevisiae* strains are still limited by the toxic compounds generated during the pretreatment process (Hasunuma et al. 2011; Sanda et al. 2011; Fujitomi et al. 2012). These toxic compounds are divided into three main groups based on origin: weak acids, furan derivatives, and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000a, b). These toxins negatively affect cell growth, metabolism, and ethanol production.

The most common weak acids present in lignocellulosic hydrolysates include acetic, formic, and levulinic acids. Acetic acid is produced by the deacetylation of hemicellulose and typically found at a higher concentration than those of other weak acids. Formic acid could be formed by the breakdown of 5-hydroxymethyl-2-furaldehyde (HMF) and furfural decomposition under acidic conditions at high temperatures (Larsson et al. 1999; Liu et al. 2012). The concentration of formic acid is typically lower than that of acetic acid but formic acid is more toxic to *S. cerevisiae* than acetic acid (Hasunuma et al. 2011). Levulinic acid is present at even lower concentrations than formic acid but it may be a more potent inhibitor than acetic acid due to its lower p*K*a value and higher lipophilicity (Larsson et al. 1999; Almeida et al. 2007; Jönsson et al. 2013). The concentrations of these weak acids in lignocellulosic hydrolysates depend on the type of biomass, pretreatment, and hydrolysis methods (Larsson et al. 1999; Palmqvist and Hahn-Hägerdal 2000b; Almeida et al. 2007; Sánchez and Cardona 2008; Liu et al. 2012).

Low concentrations of weak acids can increase the ethanol yield but high concentrations of these acids inhibit ethanol production (Larsson et al. 1999; Palmqvist et al. 1999). Based on these observations, there are two proposed mechanisms of inhibition of weak acids: uncoupling and intracellular anion accumulation (Palmqvist and Hahn-Hägerdal 2000b; Almeida et al. 2007). The undissociated form of weak acids can diffuse from the fermentation medium across the plasma membrane and dissociate due to a higher intracellular pH, decreasing the cytosolic pH. In addition, weak acids can also inhibit yeast growth by reducing the uptake of aromatic amino acids from the medium, most likely due to the strong inhibition of the Tat2p amino acid permease (Almeida et al. 2007).

To improve the inhibitor tolerance of the strain during the fermentation, metabolic, genetic and evolutionary engineering strategies have been used (Almeida et al. 2007). Deleting the gene encoding *p*-nitrophenyl phosphatase (*PHO13*) in recombinant *S. cerevisiae* strains may improve growth and ethanol production from xylose in both the presence and absence of inhibitors (Van Vleet et al. 2008; Fujitomi et al. 2012). The *TAL1* gene, encoding transaldolase that converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate, had also been studied; its over-expression again can improve the efficiency of xylose fermentation and inhibitor tolerance (Walfridsson et al. 1995; Ni et al. 2007; Bengtsson et al. 2008; Hasunuma et al. 2011).

Although genetic engineering strategies have been useful in improving the resistance of *S. cerevisiae*

strains to inhibitors, most studies use laboratory strains as starting strains and it is unclear whether the results of these studies are applicable to industrial strains. For industrial bioethanol production, selecting industrial yeast strains is very important for optimal performance under industrial conditions (Matsushika et al. 2009a, b). Many industrial strains exhibit increased tolerance to inhibitors in lignocellulosic hydrolysates compared to that of laboratory strains. In the present study, a recombinant flocculating industrial *S. cerevisiae* strain that could ferment xylose was selected as parental strain to evaluate the effects of *PHO13* deletion and *TAL1* over-expression on ethanol production in the presence of weak acid inhibitors, including formic, acetic, and levulinic acids. Most importantly, the synergistic effects of these two genes on xylose fermentation were investigated.

Materials and methods

Strains, plasmids, and primers

The xylose-utilizing recombinant industrial *S. cerevisiae* strain NAPX37 was genetically engineered from the industrial flocculent yeast strain KF-7 (Tang et al. 2006) by genome-integrating *XYL1*, *XYL2*, and *GXS1* from *Scheffersomyces stipitis* as well as *BGL1* from *Saccharomycopsis fibuligera* and over-expressing *S. cerevisiae XKS1* and *HXT7*, so the strain can metabolize xylose and cellobiose (Liu et al. 2012). The plasmids pBlu-LTKTL-TDH3 and pZeo were developed in a previous study (Tomitaka et al. 2013). *Escherichia coli* DH5α were used for plasmid preparations. The yeast strains, plasmids, and primers used in the present study are summarized in Supplementary Tables S1 and S2.

Medium

Yeast strains were routinely cultivated at 30 °C in 2 % YPD medium (20 g peptone/l, 10 g yeast extract/l, and 20 g glucose/l) with 1.5 % (w/v) agar. Each strain was cultured in spore medium (0.5 g glucose/l, 20 g potassium acetate/l, 2 g yeast extract/l, pH 5.5) for spore formation. Strains harboring the plasmid pZeo were grown on YPD/Zeo medium (2 % YPD medium containing 100 mg zeocin/l). YPG medium (20 g peptone/l, 10 g yeast extract/l, and 20 g galactose/l) was used to induce the expression of Cre recombinase from the pZeo plasmid. Batch fermentation was performed in 4 % YPX medium (20 g peptone/l, 10 g yeast extract/l, and 40 g xylose/l). *E. coli* was grown in LB medium (10 g peptone/l, 5 g yeast extract/l, and 5 g NaCl/l) containing 100 mg ampicillin/l.

Generation of haploid strain

Diploid strains were cultured in spore medium for 2–3 days, and then the tetrad was dissected using Singer MSM300 System (Singer Instruments, UK) after treated with lyticase (1 U/μl) at 30 °C for 20 min. Spores were cultured in 2 % YPD medium containing 40 mg adenine sulfate/l at 30 °C for 2–3 days, and the ploidy of cells was determined by microscope observation and spore forming test. Finally, the mating types of the cells were determined by their abilities to mate with haploid strains NAM26-15A (*MATa*) and NAM34-4C (*MATα*).

Construction of yeast strains

Fusion PCR was used to amplify the *PHO13* deletion cassette with *kanMX* as the marker. The *loxP*-P_{TEF}-*kanMX*-T_{TEF}-*loxP* fragment was amplified from the plasmid pBlu-LTKTL-TDH3 using the primers Fout and Rout, and the upstream fragment of the *PHO13* gene was amplified from the yeast genome using the primers F1 and R. The fragment for transformation (2342 bp) was amplified by fusion PCR using the primers F1 and Rout. Transformations were performed using the lithium acetate method as described previously (Tomitaka et al. 2013). The fusion fragments were transformed to the *PHO13* gene region of haploid strains NAPX37-a and NAPX37-α for homologous recombination to yield *PHO13* deleted strains NAPX37/Δ*PHO13*-a and NAPX37/Δ*PHO13*-α, respectively. Mating these two haploid strains to generate the diploid strain NAPX37/Δ*PHO13*.

The *loxP*-P_{TEF}-*kanMX*-T_{TEF}-*loxP*-P_{TDH3} fragment was amplified from the plasmid pBlu-LTKTL-TDH3 using the primers TAL1-loxP and TAL1-TDH3p harboring the sequences for homologous recombination of *TAL1* promoter. The fragments were transformed to the promoter region of *TAL1* gene of haploid strains NAPX37-a and NAPX37-α for homologous recombination to produce the *TAL1* over-expressing

strains NAPX37/*TAL1*-a and NAPX37/*TAL1*-α, respectively. The transformation was confirmed by PCR using the primers F-TAL1-UP800 and R-TAL1-DOWN200, and two haploid strains were mated to produce the diploid strain NAPX37/*TAL1*.

PHO13 deleted strains NAPX37/Δ*PHO13*-a and NAPX37/Δ*PHO13*-α were used as parental strains for generating strains with *PHO13* deletion and *TAL1* over-expression. The *kanMX* marker was firstly removed from NAPX37/Δ*PHO13*-a and NAPX37/Δ*PHO13*-α strains using the methods described by Tomitaka et al. (2013). Briefly, the strain containing the *loxP-kanMX-loxP* cassette was transformed with plasmid pZeo harboring the *cre* gene. The expression of Cre recombinase was induced by galactose in YPG medium, and then the *kanMX* marker was knocked out. The *loxP*-P_{TEF}-*kanMX*-T_{TEF}-*loxP*-P_{TDH3} was amplified from the genome of NAPX37/*TAL1* using the primers Ftal and Rtal. The fragments were transformed to the promoter region of *TAL1* gene of haploid strains NAPX37/Δ*PHO13*-a and NAPX37/Δ*PHO13*-αfor homologous recombination to produce *TAL1* over-expressing strains NAPX37/Δ*PHO13-TAL1*-a and NAPX37/Δ*PHO13-TAL1*-α, respectively. The transformation was also confirmed by PCR using the primers F-TAL1-UP800 and R-TAL1-DOWN200, and two haploid strains were mated to produce the diploid strain NAPX37/Δ*PHO13-TAL1*.

Aerobic growth kinetics

After pre-cultivation in 2 % YPD medium for 16 h, yeast cells were harvested by centrifugation at 3,000*g* at 4 °C for 6 min. The cell pellets were then washed twice with distilled water and used to inoculate 2 % YPX medium. The initial cell concentration was adjusted to an OD_{660} value of 0.05, and aerobic cultivation was performed at 30 °C with shaking at 160 rpm in a 500 ml flask. Cultivation broth was sampled periodically (2 ml) and centrifuged at 9,000*g* for 3 min. Cell pellet was washed twice with distilled water and then dispersed in 0.05 mM EDTA to measure the OD_{660}.

Fermentation assays with inhibitors

After pre-cultivation in 2 % YPD agar plate for 24 h, yeast cells were cultured under aerobic conditions at 30 °C for 16 h in 5 % YPD medium. Cells were harvested by centrifugation at 3,000*g* at 4 °C for 6 min, and washed twice with distilled water. Cell pellet was inoculated to 4 % YPX fermentation medium containing the inhibitors, formic acid, acetic acid, or levulinic acid. The initial cell concentration was adjusted to 50 g wet cell/l (10 g/l dry cell weight), and the fermentation was performed at 35 °C for 48 h with an agitation speed of 200 rpm in a 300 ml flask. The agitation speed was controlled by a HS-6DN magnetic stirrer (As One, Japan).

Measurement of fermentation products

Xylose in the fermentation medium was determined by a HPLC equipped with a fluorescence detector (RF-$10A_{XL}$) as previously described (Tang et al. 2006). Ethanol was measured by GC with an FID detector. 2-propanol was used as the internal standard (Tang et al. 2006). Xylitol and glycerol were assayed by HPLC equipped with an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, USA) and a refractive index detector. The HPLC was operated at 65 °C, with a mobile phase of 5 mM H_2SO_4, at 0.6 ml/min with an injection of 50 μl.

Results and discussion

Aerobic growth kinetics

The diploid strains, NAPX37/Δ*PHO1*3, NAPX37/*TAL1*, and NAPX37/Δ*PHO13-TAL1* (Supplementary Table S1) constructed using strain NAPX37 as the parental strain. The growth of the parental and engineered strains in 2 % YPX medium under aerobic conditions were investigated (Fig. 1). Although the growth of the parental strain NAPX37 was slower than that of the other three strains, the difference was not significant. Strain NAPX37/Δ*PHO13-TAL1* entered the stationary phase first. The times of the growth phases for NAPX37/Δ*PHO13* and NAPX37/*TAL1* were almost identical, but the biomass of the latter was higher. However, the biomass of both of them were greater than that of the parental strain NAPX37. These results indicate that *PHO13* deletion or *TAL1* over-expression has positive effects on cell growth, which is in accordance with previous studies, but the effects were not as significant compared to those of previous studies (Walfridsson et al. 1995; Ni et al. 2007; Van

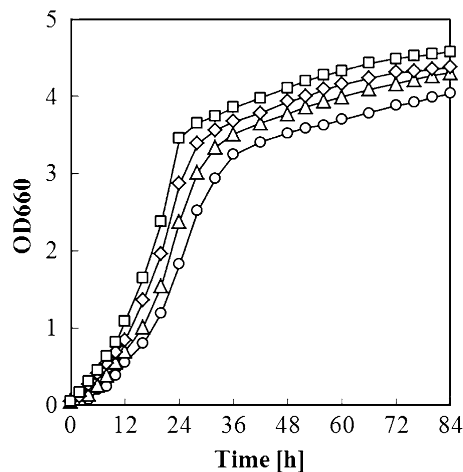

Fig. 1 Aerobic growth kinetics of the engineered strains. The initial cell concentration was adjusted to an OD_{660} of 0.05. *Symbols* NAPX37 (*circles*), NAPX37/*ΔPHO13* (*triangles*), NAPX37/*TAL1* (*diamonds*), and NAPX37/*ΔPHO13-TAL1* (*squares*). Values represent the averages of duplicate experiments, and the standard deviations (SD) were <5 % of the mean

Vleet et al. 2008; Fujitomi et al. 2012). The differences in genetic background of these strains could explain the different effects of *PHO13* deletion or *TAL1* over-expression. The parental strain NAPX37 exhibits good growth characteristics when cultivated in medium with xylose as its sole carbon source.

Xylose fermentation in the absence of inhibitors

The xylose fermentation ability of the four diploid strains NAPX37, NAPX37/*ΔPHO13*, NAPX37/*TAL1*, and NAPX37/Δ*PHO13-TAL1* under O_2 limited conditions at 35 °C was evaluated. The initial cell concentration was adjusted to 10 g DCW/l. As shown in Fig. 2, the four strains nearly consumed all xylose within 12 h. NAPX37/Δ*PHO13-TAL1* produced the highest amount of ethanol and exhibited a slight decrease in the accumulation of xylitol after 48 h. The maximum xylose consumption rate and specific ethanol productivity were 6.22 g xylose/g DCW/h and 1.81 g ethanol/g DCW/h, respectively (Table 1). However, they were 5.31 g xylose/g DCW h and 1.54 g ethanol/g DCW/h for the control strains NAPX37, respectively. The strains NAPX37/*ΔPHO13* and NAPX37/*TAL1* displayed moderate fermentation ability, but the xylose consumption rate and ethanol productivity of the former was lower than those of the latter (Table 1). Although all strains displayed low glycerol yields, the strain NAPX37/*TAL1* still produced the lowest amount of glycerol (Fig. 2).

These results indicated that *PHO13* deletion or *TAL1* over-expression in strain NAPX37 could improve xylose consumption and ethanol production. According to previous studies, *PHO13* deletion or *TAL1* over-expression had good effects on ethanol production for *S. cerevisiae* strains (Ni et al. 2007; Van Vleet et al. 2008; Fujitomi et al. 2012; Sanda et al. 2011).

TAL1 over-expression can improve the efficiency of xylose consumption and fermentation of *S. cerevisiae* (Walfridsson et al. 1995; Ni et al. 2007; Bengtsson et al. 2008; Hasunuma et al. 2011). Deleting or reducing *PHO13* expression in *S. cerevisiae* strains also enhanced the fermentation of xylose (Ni et al. 2007; Van Vleet et al. 2008; Fujitomi et al. 2012). However, the synergistic effects of these two genes on xylose fermentation are still unclear. In the present study, the simultaneous deletion of the *PHO13* gene and over-expression of *TAL1* gene could improve ethanol production and decrease the accumulation of xylitol of industrial *S. cerevisiae*.

Xylose fermentation in the presence of formic acid

Xylose fermentation in the presence of 15 and 30 mM formic acid was investigated (Fig. 3). Fermentation was significantly inhibited with an increase in the concentration of formic acid. In the presence of 15 mM formic acid, four strains exhibited good xylose fermentation capabilities. The parental strain NAPX37 produced 10.9 g ethanol/l with 33.8 g xylose consumption, whereas the strains NAPX37/*ΔPHO13*, NAPX37/*TAL1*, and NAPX37/Δ*PHO13-TAL1* generated 11.3, 11.5, and 12.3 g ethanol/l, respectively (Fig. 3). In addition, the xylitol yields of NAPX37/*ΔPHO13*, NAPX37/*TAL1*, and NAPX37/Δ*PHO13-TAL1* strains decreased by 29, 25, and 36 %, respectively, compared to that of the parental strain (Table 1). However, when formic acid was increased to 30 mM, only 9.22 g xylose was consumed by the parental strain NAPX37 and 1.4 g ethanol/l was produced. *PHO13* deletion or *TAL1* over-expression could enhance the ethanol yield slightly, and the yield was further enhanced by the simultaneous deletion and over-expression of these two genes. Strain NAPX37/Δ*PHO13-TAL1* produced 2.67 g ethanol/l after 48 h fermentation, which is 1.3-, 1.5-, and 1.9-fold higher

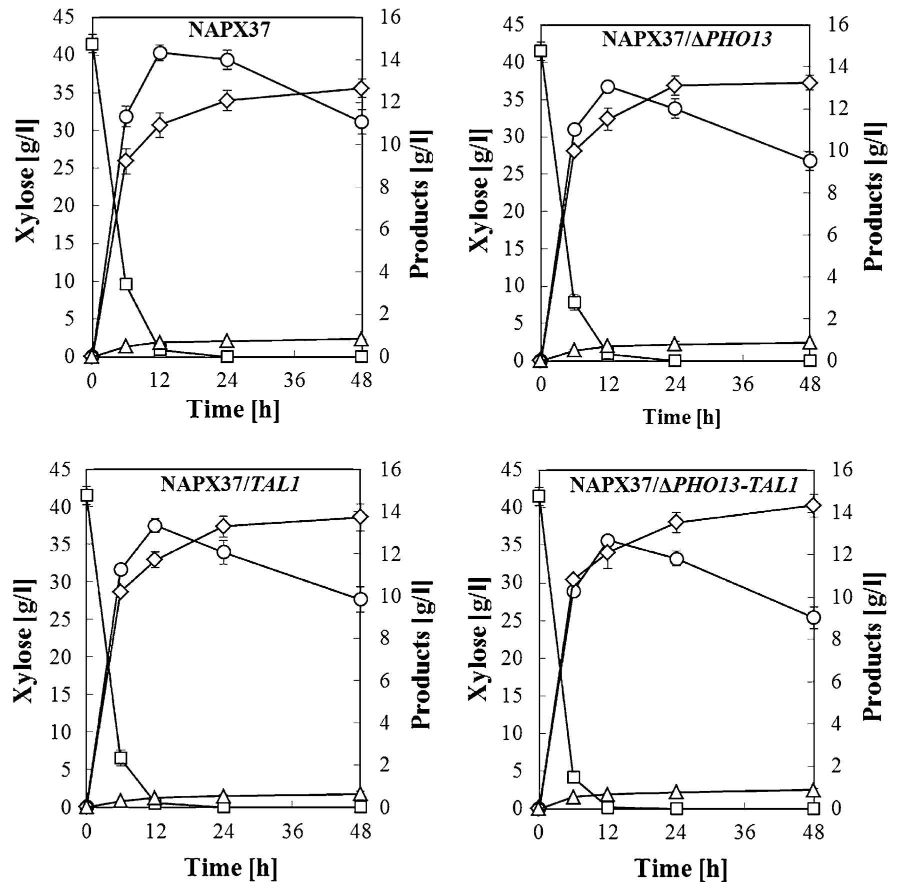

Fig. 2 Xylose fermentation capability of engineered strains under the condition without inhibitors. The initial cell concentration was 10 g DCW/l. *Symbols* xylose (*squares*), ethanol (*diamonds*), xylitol (*circles*) and glycerol (*triangles*). Values represent the averages of duplicate experiments ± SD

than that of NAPX37/*TAL1*, NAPX37/*ΔPHO13*, and NAPX37, respectively. These results indicate that simultaneously over-expressing *TAL1* and deleting *PHO13* has synergistic effects on improving the resistance of *S. cerevisiae* strains to formic acid during the production of ethanol from xylose.

Formic acid is the most toxic weak acid in lignocellulosic hydrolysates due to its low molecular weight and lower p*K*a value (Larsson et al. 1999; Almeida et al. 2007; Jönsson et al. 2013). In the present study, the initial pH of the fermentation medium may be an important factor affecting the performance of the strains, as another study in our laboratory showed that the fermentation ability of the strain NAPX37 could be significantly improved when the medium pH was adjusted to 5 even though formic acid was increased to 80 mM (data not shown). A laboratory xylose-fermenting *S. cerevisiae* strain over-expressing *TAL1* or with the deletion of *PHO13* improved ethanol yields when exposed to formic acid (Sanda et al. 2011; Fujitomi et al. 2012). The ethanol yield of strains with *PHO13* deletion or *TAL1* over-expression decreased slightly compared to that of the parental strain used in this study, whereas the ethanol yield of the strain over-expressing *TAL1* with the *PHO13* deletion improved, indicating that these two genes exhibited synergistic effects on xylose fermentation of the industrial *S. cerevisiae* strain in the presence of formic acid.

Xylose fermentation in the presence of acetic acid

The effects of acetic acid (30 and 60 mM) on the four engineered *S. cerevisiae* strains were evaluated (Fig. 4). Xylose consumption in the four strains significantly decreased with the increase of acetic acid concentration. Xylose was almost completely metabolized within 24 h in the presence of 30 mM acetic acid. However, its concentration after 48 h fermentation in the presence of 60 mM acetic acid was almost

Table 1 Fermentation characteristics of the recombinant *S. cerevisiae* strains under different conditions

Strain	Inhibitor	Conc. (mM)	Initial pH of fermentation	Yield (g/g-xylose)[a]		r_{max} (h^{-1})[b]	P_{max} (h^{-1})[c]
				Ethanol	Xylitol		
NAPX37	Without inhibitor	–	5.6	0.31 ± 0.01	0.27 ± 0.01	5.31 ± 0.11	1.54 ± 0.04
	Formic acid	15	4.2	0.32 ± 0.01	0.26 ± 0.01	1.97 ± 0.06	0.64 ± 0.07
		30	3.8	0.15 ± 0.01	0.25 ± 0.02	0.74 ± 0.04	0.18 ± 0.00
	Acetic acid	30	4.3	0.30 ± 0.01	0.27 ± 0.00	2.62 ± 0.11	0.91 ± 0.08
		60	4.0	0.28 ± 0.00	0.18 ± 0.01	1.20 ± 0.12	0.38 ± 0.01
	Levulinic acid	15	4.5	0.30 ± 0.01	0.27 ± 0.01	2.98 ± 0.07	1.04 ± 0.02
		30	4.2	0.29 ± 0.01	0.26 ± 0.01	2.06 ± 0.09	0.73 ± 0.05
NAPX37/Δ*PHO13*	Without inhibitor		5.6	0.32 ± 0.01	0.23 ± 0.01	5.61 ± 0.15	1.67 ± 0.03
	Formic acid	15	4.2	0.31 ± 0.01	0.18 ± 0.02	2.20 ± 0.09	0.67 ± 0.03
		30	3.8	0.16 ± 0.01	0.18 ± 0.01	1.01 ± 0.06	0.20 ± 0.01
	Acetic acid	30	4.3	0.32 ± 0.01	0.22 ± 0.01	2.96 ± 0.17	1.10 ± 0.08
		60	4.0	0.29 ± 0.01	0.14 ± 0.01	1.37 ± 0.09	0.42 ± 0.01
	Levulinic acid	15	4.5	0.31 ± 0.00	0.23 ± 0.00	3.34 ± 0.14	1.28 ± 0.04
		30	4.2	0.29 ± 0.01	0.20 ± 0.01	2.16 ± 0.11	0.76 ± 0.02
NAPX37/*TAL1*	Without inhibitor		5.6	0.33 ± 0.02	0.24 ± 0.01	5.83 ± 0.23	1.70 ± 0.06
	Formic acid	15	4.2	0.31 ± 0.02	0.19 ± 0.01	2.22 ± 0.09	0.69 ± 0.04
		30	3.8	0.16 ± 0.01	0.15 ± 0.00	1.16 ± 0.07	0.23 ± 0.06
	Acetic acid	30	4.3	0.32 ± 0.01	0.22 ± 0.01	3.16 ± 0.18	1.12 ± 0.03
		60	4.0	0.29 ± 0.01	0.15 ± 0.01	1.45 ± 0.04	0.47 ± 0.01
	Levulinic acid	15	4.5	0.32 ± 0.02	0.23 ± 0.01	3.29 ± 0.15	1.24 ± 0.07
		30	4.2	0.31 ± 0.02	0.21 ± 0.00	2.36 ± 0.07	0.77 ± 0.03
NAPX37/Δ*PHO13*-*TAL1*	Without inhibitor		5.6	0.35 ± 0.01	0.22 ± 0.00	6.22 ± 0.18	1.81 ± 0.05
	Formic acid	15	4.2	0.33 ± 0.02	0.17 ± 0.01	2.34 ± 0.12	0.80 ± 0.06
		30	3.8	0.18 ± 0.01	0.12 ± 0.01	1.39 ± 0.05	0.28 ± 0.00
	Acetic acid	30	4.3	0.35 ± 0.02	0.21 ± 0.01	3.30 ± 0.13	1.15 ± 0.02
		60	4.0	0.29 ± 0.01	0.13 ± 0.01	1.67 ± 0.08	0.51 ± 0.02
	Levulinic acid	15	4.5	0.34 ± 0.01	0.23 ± 0.01	3.70 ± 0.16	1.33 ± 0.05
		30	4.2	0.32 ± 0.01	0.19 ± 0.02	2.39 ± 0.13	0.81 ± 0.04

Values are averages of duplicate experiments ± SD

[a] *Yield* yields are based on consumed xylose after 48 h fermentation

[b] r_{max} maximum specific xylose consumption rate (g xylose/g DCW/h)

[c] P_{max} maximum specific ethanol productivity (g ethanol/g DCW/h)

[b,c] Maximum specific xylose consumption rate, maximum specific ethanol productivity were calculated from the data in the first 6 h

the same as that after 12 h fermentation in the presence of 30 mM acetic acid. Although the specific xylose consumption of the strain with *PHO13* deletion or *TAL1* over-expression was slightly different under the same conditions (Table 1), the strain NAPX37/*ΔPHO13* and NAPX37/*TAL1* displayed better xylose fermentation capabilities compared with that of the parental strain. The ethanol yields of these two strains at 30 mM acetic acid were 0.34 g/g xylose and 0.32 g/g xylose, respectively. At 60 mM acetic acid, the ethanol yields of NAPX37/*ΔPHO13* and NAPX37/*TAL1* were both 0.29 g/g xylose. The accumulation of xylitol by the strains NAPX37/*ΔPHO13* and NAPX37/*TAL1* was also lower than that of the parental strain but the yield of the former was less than the latter in the presence of the same concentration of acetic acid. Among all of the strains, NAPX37/Δ*PHO13-TAL1* effectively consumed the most xylose and produced

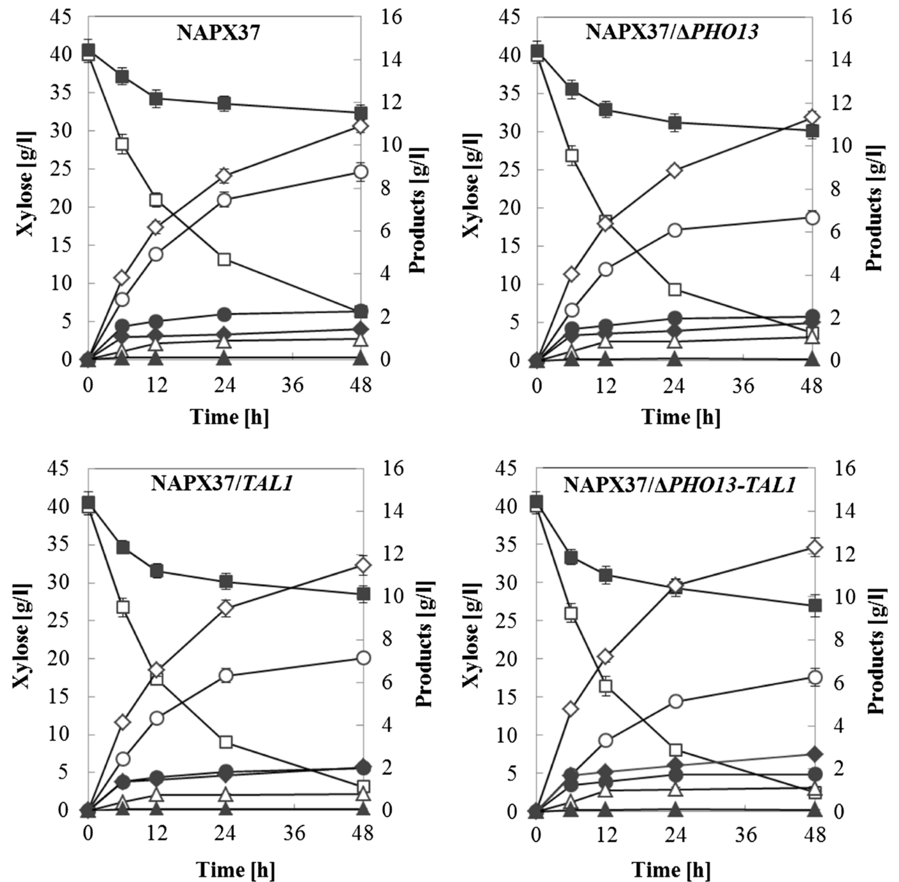

Fig. 3 Comparison of xylose fermentation capability of engineered strains in the presence of 15 mM (*open symbols*) and 30 mM (*closed symbols*) formic acid. The initial cell concentration was 10 g DCW/l. *Symbols* xylose (*squares*), ethanol (*diamonds*), xylitol (*circles*), and glycerol (*triangles*). Values represent the averages of duplicate experiments ± SD

more ethanol, regardless of the concentration of acetic acid. The maximum specific xylose consumption rate and specific ethanol productivity of NAPX37/Δ*PHO13-TAL1* were 3.3 g xylose/g DCW/h and 1.15 g ethanol/g DCW/h, respectively, at 30 mM acetate (Table 1). These values were both 1.26-fold higher than those of the parental strain NAPX37. Furthermore, when the acetic acid concentration was increased to 60 mM, these values were 1.45-fold and 1.32-fold higher, respectively (Table 1).

Acetic acid is the most prominent weak acid in lignocellulosic hydrolysates and has a significant effect on xylose fermentation of *S. cerevisiae* strain (Almeida et al. 2007). Both *TAL1* over-expression and *PHO13* deletion can effectively improve the tolerance of *S. cerevisiae* strains to acetic acid (Hasunuma et al. 2011; Fujitomi et al. 2012). Our results are consistent with these findings, with the exception that this improvement is not significant (Table 1). Although Fujitomi et al. (2012) reported that the ethanol yield of a *PHO13*-deleted strain was slightly higher than that of a *TAL1*-over-expressing strain, our findings indicate that *TAL1* over-expression seems to be conducive to producing more ethanol. These differences may be caused by the different genetic backgrounds of the strains used in each study. The parental strain NAPX37 used in this study originated from an industrial strain and has resistant to inhibitors during the fermentation of lignocellulosic hydrolysates (Liu et al. 2012). Furthermore, our results also indicated that the simultaneous over-expression of *TAL1* and deletion of *PHO13* exhibited synergistic effects on xylose fermentation for industrial *S. cerevisiae* strains in the presence of acetic acid.

Xylose fermentation in the presence of levulinic acid

Xylose fermentation in the presence of 15 and 30 mM levulinic acid was investigated (Fig. 5). In the presence of 15 mM levulinic acid, all strains consumed xylose almost completely after 24 h. The parental

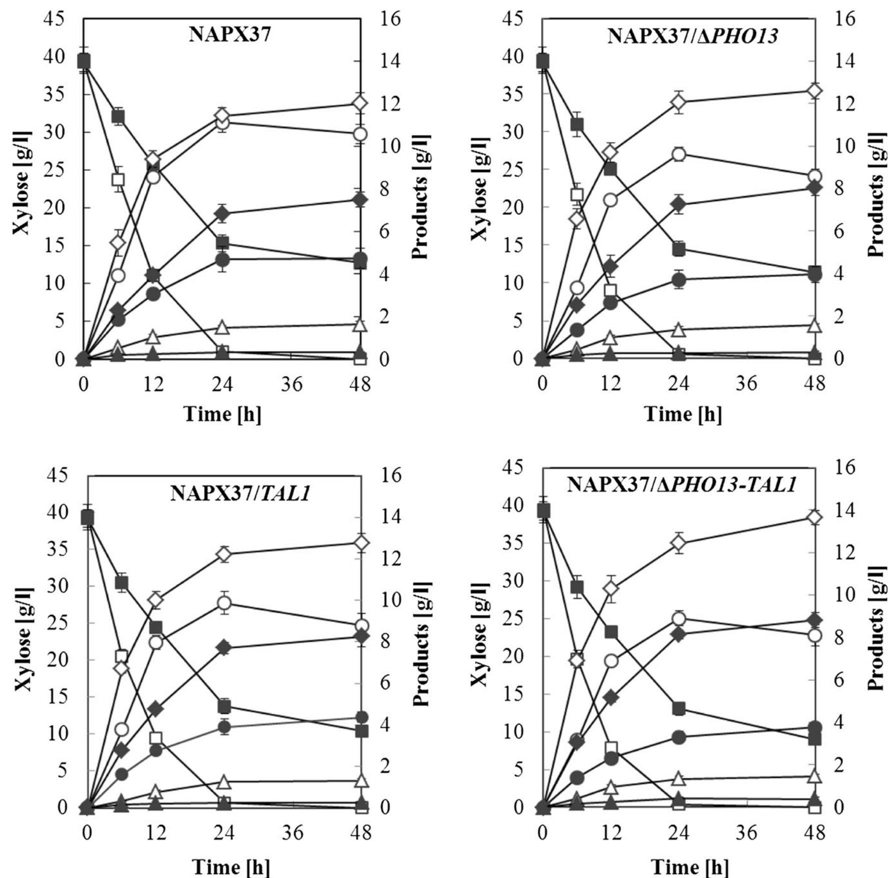

Fig. 4 Comparison of xylose fermentation capability of engineered strains in the presence of 30 mM (*open symbols*) and 60 mM (*closed symbols*) acetic acid. The initial cell concentration was 10 g DCW/l. *Symbols* xylose (*squares*), ethanol (*diamonds*), xylitol (*circles*), and glycerol (*triangles*). Values represent the averages of duplicate experiments ± SD

strain NAPX37 produced 11.8 g ethanol/l and 14 g xylitol/l, respectively (Fig. 5), whereas NAPX37/*ΔPHO13* and NAPX37/*TAL1* had higher yields of ethanol and lower yields of xylitol (Table 1). This trend was further enhanced by the strain NAPX37/Δ*PHO13-TAL1*, which produced 1.1-fold more ethanol and accumulated 81 % xylitol, compared to that of the parental strain. However, when levulinic acid was increased to 30 mM, the consumption of xylose by the four strains decreased. However, only a few grams of xylose were not consumed after 48 h (Fig. 5). Strain NAPX37 produced 10.6 g ethanol/l, and the ethanol yield of NAPX37/Δ*PHO13-TAL1* increased to 0.32 g/g xylose compared to 0.29 g/g xylose of NAPX37. In addition, NAPX37 accumulated 9.26 g xylitol/l after 48 h fermentation, whereas the xylitol yields of NAPX37/*ΔPHO13*, NAPX37/*TAL1*, and NAPX37/Δ*PHO13-TAL1* decreased 20, 18, and 27 %, respectively. Additionally, all strains displayed poor glycerol production but the *TAL1*-over-expressing strain produced the lowest amount of glycerol.

Levulinic acid is formed by the degradation of 5-HMF during lignocellulosic hydrolysis, and its concentration is related to the method of biomass hydrolysis (Liu et al. 2012). Few studies have investigated the effect of this inhibitor on xylose fermentation by industrial *S. cerevisiae* strains. Levulinic acid is a stronger inhibitor than acetic acid which may be due to its lower p*K*a values and higher lipophilicity (Larsson et al. 1999; Almeida et al. 2007; Jönsson et al. 2013). Here, we found that xylose consumption by strain NAPX37 after 12 h fermentation in the presence of 30 mM acetic acid is almost the same as its consumption after 24 h fermentation in the presence of 30 mM levulinic acid. However, ethanol produced in 30 mM acetic acid was 1.3-fold higher than ethanol in the same concentration of levulinic acid. Furthermore, our results also suggested that *TAL1* over-expression and *PHO13* deletion exhibited synergistic effects which leading to improved ethanol production during xylose fermentation of industrial *S. cerevisiae* strains in the presence of levulinic acid.

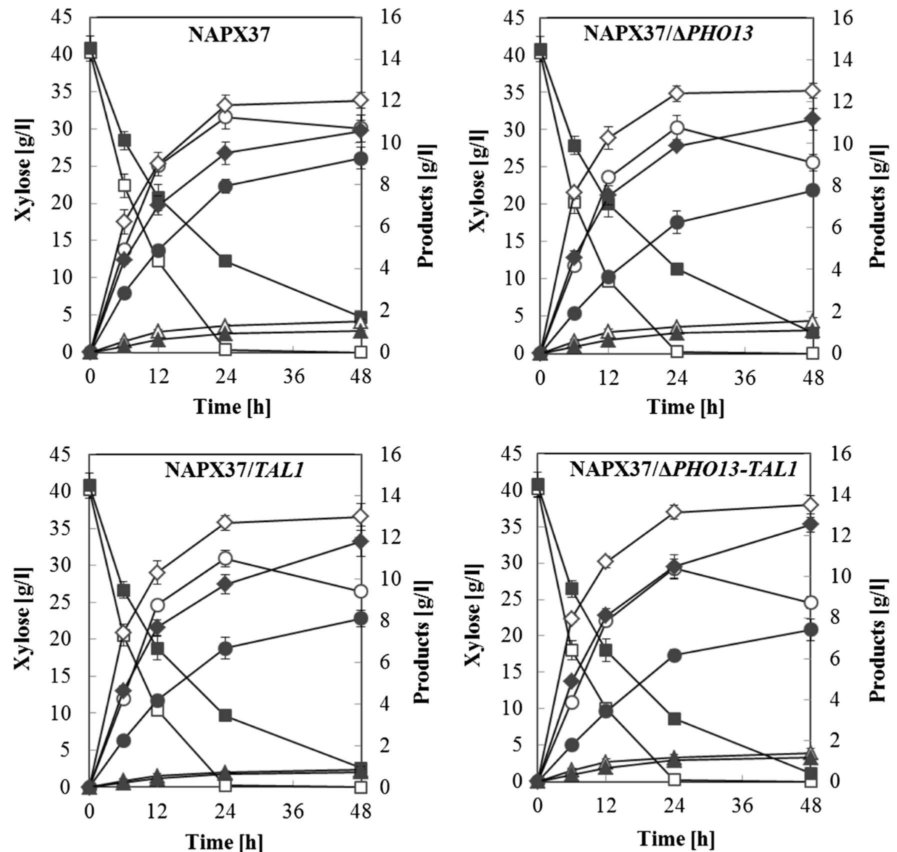

Fig. 5 Comparison of xylose fermentation capability of engineered strains in the presence of 15 mM (*open symbols*) and 30 mM (*closed symbols*) levulinic acid. The initial cell concentration was 10 g DCW/l. *Symbols* xylose (*squares*), ethanol (*diamonds*), xylitol (*circles*), and glycerol (*triangles*). Values represent the averages of duplicate experiments ± SD

Conclusions

Either *TAL1* over-expression or *PHO13* deletion in the industrial-flocculating *S. cerevisiae* strain NAPX37 could improve cell growth and promote xylose consumption, which increasing the ethanol production in the absence of weak acids. In the presence of formic, acetic, or levulinic acids, the engineered strains produced more ethanol and accumulated less xylitol. The results also indicated that inhibition by levulinic acid is stronger than that of acetic acid but weaker than that of formic acid. The inhibition of weak acids also enhanced with the increase of inhibitor concentration. Most importantly, this study demonstrates that the simultaneous over-expression of *TAL1* and deletion of *PHO13* had synergistic effects on xylose fermentation of industrial *S. cerevisiae* strain in the presence of weak acids. However, further researches on the tolerance of other inhibitors including furans and aldehydes should be conducted to make the strain able to ferment lignocellulosic hydrosates with high contents of various inhibitors.

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