

Deleting the *para*-nitrophenyl phosphatase (pNPPase), *PHO13*, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose

Jennifer Headman Van Vleet^{a,b}, Thomas W. Jeffries^{b,c}, Lisbeth Olsson^{a,*}

^aCenter for Microbial Biotechnology, Technical University of Denmark, Biocentrum-DTU, Building 223, DK-2800 Kgs. Lyngby, Denmark

^bDepartment of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706, USA

^cForest Products Laboratory, Forest Service, USDA, Madison, WI 53725-2398, USA

Received 5 September 2007; received in revised form 27 November 2007; accepted 7 December 2007

Available online 27 December 2007

Abstract

Overexpression of D-xylulokinase in *Saccharomyces cerevisiae* engineered for assimilation of xylose results in growth inhibition that is more pronounced at higher xylose concentrations. Mutants deficient in the *para*-nitrophenyl phosphatase, *PHO13*, resist growth inhibition on xylose. We studied this inhibition under aerobic growth conditions in well-controlled bioreactors using engineered *S. cerevisiae* CEN.PK. Growth on glucose was not significantly affected in *pho13Δ* mutants, but acetate production increased by 75%. Cell growth, ethanol production, and xylose consumption all increased markedly in *pho13Δ* mutants. The specific growth rate and rate of specific xylose uptake were approximately 1.5 times higher in the deletion strain than in the parental strain when growing on glucose–xylose mixtures and up to 10-fold higher when growing on xylose alone. In addition to showing higher acetate levels, *pho13Δ* mutants also produced less glycerol on xylose, suggesting that deletion of Pho13p could improve growth by altering redox levels when cells are grown on xylose.

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Keywords: *PHO13*; Fermentation; Overexpression; Xylulokinase; Xylose; *XYL3*; *XKS1*; *XYL1*; *XYL2*

1. Introduction

Lignocellulose from forest and agricultural products is an inexpensive and readily available feedstock for bioethanol production. Current mature bioethanol technologies are crop based and utilize substrates such as corn starch and sugarcane juice. In order to make the conversion of lignocellulosic biomass to useable energy economical and environmentally friendly, both cellulose and hemicellulose must be used. Hemicellulose is composed of hexose sugars, such as glucose and mannose, and pentose sugars such as xylose and arabinose. Xylose is the predominant sugar in angiosperm hemicellulose, and as such is the second most abundant carbohydrate in nature after glucose. Development of an improved microbe capable of fermenting xylose

to ethanol on a large scale could greatly reduce the overall cost of bioconversion (Hahn-Hagerdal et al., 2007; Jeffries, 2006; Zaldivar et al., 2001).

The use of *Saccharomyces cerevisiae* in ethanol fermentations has several advantages. Knowledge of its physiology is extensive and a large number of genetic tools are available for its manipulation. Furthermore, it has a long history as ethanol producer and its fermentation technology is well established. It is also ethanol tolerant and generally regarded as being safe. Native *S. cerevisiae*, however, is unable to grow on xylose. There are two possible pathways that can be used when developing a strain of *S. cerevisiae* that is capable of growing on xylose. The first incorporates the xylose metabolic pathway from a xylose utilizing yeast. In one such yeast, *Pichia stipitis*, conversion of xylose to xylulose is catalyzed in two steps. First, xylose is reduced to xylitol by an NAD[P]H + H⁺ linked xylose reductase (*PsXYL1*), and xylitol is oxidized

*Corresponding author. Fax: +45 4588 4148.

E-mail address: lo@biocentrum.dtu.dk (L. Olsson).

to xylulose by a NAD^+ linked xylitol dehydrogenase (*PsXYL2*) (Verduyn et al., 1985). The second pathway involves expression of a fungal or bacterial xylose isomerase that converts xylose directly to xylulose (Kuyper et al., 2004). Regardless of the pathway chosen for engineering, xylulose must then be converted to xylulose-5-phosphate by a D-xylulokinase before it enters the pentose phosphate pathway.

S. cerevisiae contains a gene for D-xylulokinase (*ScXKS1*), but its native level of expression is too low to allow strong growth and fermentation on xylulose. Studies by Jin and Jeffries (2003) and Jin et al. (2002) found that recombinant *S. cerevisiae* transformed with a single copy of *PsXYL1* and multiple copies of *PsXYL2* accumulated xylulose, which suggests that the native level of xylulokinase limits xylose assimilation when *XYL1* or *XYL2* are overexpressed. A study transforming a genome fragment library from *P. stipitis* into recombinant *S. cerevisiae* expressing *XYL1* and *XYL2* supported this conclusion. Ten out of 16 transformants identified as having a higher specific growth rate on xylose contained the *PsXYL3* gene (Jin et al., 2005). Also, Wahlbom et al. (2003a, b) reported increased xylulokinase activity in a mutant strain displaying an improved ability to utilize xylose.

The level of xylulokinase activity necessary for effective utilization of xylose has been the subject of much discussion and research. Early reports by Ho et al. (1998) indicated that simultaneously overexpressing the *S. cerevisiae* endogenous xylulokinase (*ScXKS1*) along with the xylose reductase and xylitol dehydrogenase from *P. stipitis* (*PsXYL1* and *PsXYL2*) increased ethanol production while decreasing xylitol production from xylose. Conversely, Rodriguez-Pena et al. (1998) showed that overexpressing *ScXKS1* completely inhibited growth on pure D-xylulose; however, a level of *ScXKS1* expression slightly higher than wild type led to improved growth. Studies by Toivari et al. (2001) and Richard et al. (2000) did not find an inhibitory effect following *ScXKS1* overexpression, but Johansson et al. (2001) found that the overexpression of *ScXKS1* reduced the specific rate of xylose consumption by 50% to 80% in *S. cerevisiae* transformants, as well as overall xylose consumption. Lee et al. (2003) reported that when comparing engineered strains of *S. cerevisiae* grown on xylulose, the strain with the highest specific xylulokinase activity had the lowest specific growth rate, and the specific growth rate and dry cell mass increased as the xylulokinase activity decreased. A study by Jin et al. (2003) showed that strong overexpression of the *P. stipitis* gene for D-xylulokinase (*PsXYL3*) also inhibited growth on xylose while moderate expression enabled optimal growth. Notably, the *P. stipitis* Xyl3p has about three times higher specific activity than Xks1p, so this finding suggests that strong overexpression of xylulokinase activity inhibits growth (Jin et al., 2003).

A recent study by Ni et al. (2007) showed that spontaneous or induced mutations in *S. cerevisiae* could overcome the inhibition caused by strong overexpression of

ScXKS1 or *PsXYL3*. Two prominent mutational events found in this study were up-regulation of *TAL1* and inactivation of *PHO13*. Although previous studies had already established the importance of *TAL1* overexpression in the fermentation of xylose and arabinose (Becker and Boles, 2003), the *PHO13* discovery was new. The engineered *S. cerevisiae* L2612 strain, in which that research was conducted, showed severely impaired growth in defined minimal medium, even when the known auxotrophies are supplemented (data not shown). This raised the possibility that other deficiencies in the L2612 genetic background might be responsible in part for the observed growth inhibition with xylulokinase overexpression on xylose. The present research investigated the effects of the *PHO13* deletion when xylulokinase is overexpressed in a well-studied genetic background, which is a preferred strain background for physiological studies.

The conversion of xylulose to xylulose-5-phosphate is an important step in the utilization of xylose for bioethanol production regardless of the pathway chosen for conversion of xylose to xylulose. Development of a yeast that is capable of growing on xylose, while expressing higher levels of xylulokinase, could increase flux through the pentose phosphate pathway and lead to higher and more efficient ethanol production from lignocellulosic biomass.

2. Materials and methods

2.1. Strains and strain construction

All *S. cerevisiae* strains used in this study, shown in Table 1, were generated from the CEN.PK 113-7D haploid wild type strain and the *ura3* auxotroph CEN.PK 113-5D (Entian and Kötter, 2007). The *PHO13* knockout cassette was liberated from the P43933 deletion plasmid by cleavage with *NotI* and purified using gel electrophoresis. P43933 was obtained from EUROSCARF (Winzeler et al., 1999). All yeast transformations were performed using a lithium acetate/single stranded carrier DNA/polyethylene glycol method with slight modifications (Gietz and Woods, 2002). Strains described as overexpressing the *P. stipitis* *XYL1*, *XYL2*, and *XYL3* genes were transformed with pYES2-X123 (Ni et al., 2007).

2.2. Drop tests

Drop tests were performed on synthetic complete media lacking uracil (Kaiser et al., 1994). *P. stipitis* CBS6054 was used as a positive growth control and distilled water was used as a negative control. Briefly, cultures were pelleted, washed with distilled water, and resuspended to a starting OD of 0.1. Four subsequent five-fold dilutions were then made and 5 μl of each dilution was dropped onto plates containing 2% (w/v) glucose, 2% (w/v) xylose, 6% (w/v) glucose, and 6% (w/v) xylose, respectively. Plates were checked daily for five consecutive days.

Table 1
Plasmids and yeast strains used

Name	Description	Source
<i>Plasmids</i>		
P43933	<i>PHO13</i> deletion plasmid	EUROSCARF (Winzeler et al., 1999)
pYES2-X123	<i>URA3</i> , 2 μ m origin, <i>TDH3_p-PsXYL1-TDH3_T TDH3_p-PsXYL2-TDH3_T TDH3_p-PsXYL3-TDH3_T</i>	Ni et al. (2007)
<i>Strains</i>		
<i>S. cerevisiae</i> CEN.PK.113-7D	<i>MATa MAL2-8^c SUC2</i>	Entian and Kötter (2007)
<i>S. cerevisiae</i> CEN.PK.113-5D	<i>MATa MAL2-8^c SUC2 ura3-52</i>	Entian and Kötter (2007)
<i>S. cerevisiae</i> CMB.JHV.XYL123	CEN.PK.113-5D (pYES2-X123)	This study
<i>S. cerevisiae</i> CMB.JHV.pho13a	Isogenic of CEN.PK.113-5D except for <i>pho13::kanMX4</i>	This study
<i>S. cerevisiae</i> CMB.JHV.XYL123.pho13a	Isogenic of CMB.JHV.XYL123 except for <i>pho13::kanMX4</i>	This study
<i>S. cerevisiae</i> CMB.JHV.pho13b	Isogenic of CEN.PK.113-7D except for <i>pho13::kanMX4</i>	This study

2.3. Medium preparation

A defined minimal medium containing trace metal elements and vitamins was used in all bioreactor cultivations. It had the following composition: 5 g (NH₄)₂ SO₄ L⁻¹, 3 g KH₂ PO₄ L⁻¹, 0.5 g MgSO₄ 7H₂O L⁻¹, 1 ml trace element solution L⁻¹, 1 ml vitamin solution L⁻¹, and 0.05 ml antifoam 289 (Sigma A-8436) L⁻¹ (Verduyn et al., 1992). For glucose cultivations, a starting concentration of 20 g L⁻¹ glucose was used. For mixed sugar cultivations, starting concentrations of 10 g L⁻¹ of each sugar were used. All xylose cultivations were performed with starting concentrations of 20 g L⁻¹ xylose.

2.4. Aerobic batch cultivations

Aerobic cultivations were performed in well-controlled baffled 5-L bioreactors with working volumes of 4 L each. The bioreactors were equipped with two disc-turbine impellers rotating at 750 rpm. Bioreactor temperature was controlled at 30 °C and the pH was kept constant at 5.0 by automatic addition of 3 N KOH. Airflow into the bioreactors was 1 vvm and the off gas was led through a condenser cooled to 4 °C to minimize evaporation of ethanol. Carbon dioxide concentrations in the exhaust gas were determined by a Brüel and Kjær 1308 acoustic gas analyzer (Brüel and Kjær, Naerum, Denmark, Christensen et al., 1995/1998). Cultivations were performed in triplicate.

2.5. Batch cultivations under microaerobic conditions

Cultivations using low aeration levels were performed in Braun Biostat B bioreactors with working volumes of 2 L each. Cultivations were performed at 30 °C with a stirrer speed of 500 rpm and the pH was kept constant at 5.0 by

the automatic addition of 5 N KOH. Air and nitrogen were mixed to an initial oxygen concentration of between 2% and 4% O₂ in the in-going gas, and cultivations were sparged at a rate of 1 vvm. Exhaust gas was cooled in a condenser to 4 °C to minimize evaporation of ethanol. Carbon dioxide concentrations in the exhaust gas were determined by a Brüel and Kjær 1308 acoustic gas analyzer (Brüel and Kjær, Naerum, Denmark, Christensen et al., 1995). Cultivations were performed in duplicate.

2.6. Cell mass determination

The dry weight was determined using polyethersulfone filters with a pore size of 0.45 μ m (Sartorius). The filters were pre-dried in a microwave oven at 150 W for 10 min, cooled in a desiccator, and weighed. A known volume of cell culture was filtered and the residue was washed with distilled water and dried on the filter for 15 min in a microwave oven at 150 W. Filters were once again cooled in a desiccator and then weighed to determine the cell mass concentration. The optical density was determined at 600 nm using a Shimadzu UV mini-1240 (Kyoto, Japan). Samples were diluted with water to obtain OD measurements in the linear range of 0.1–0.7 OD units.

2.7. Analysis of extracellular metabolites

Samples harvested from the bioreactors were immediately filtered through a 0.45 μ m pore-size cellulose acetate filter (Sartorius) and stored at –20 °C until analysis. Glucose, xylose, xylitol, glycerol, ethanol, acetate, and pyruvate concentrations were determined by HPLC analysis using an Aminex HPX-87H column from Biorad. The column was kept at 65 °C and eluted at 0.6 ml min⁻¹ with 5 mM H₂SO₄. The column was coupled to two detectors connected in series: a Waters 410 Differential Refractometer (Millipore, CA, USA) for glucose, xylose,

xylitol, ethanol, and glycerol determination and a Waters 486 Tunable Absorbance Detector set at 210 nm for acetate and pyruvate determination (Roca and Olsson, 2003).

3. Results

3.1. Confirmation of the growth inhibition phenotype

S. cerevisiae CMB.JHV.XYL123 was not able to grow on plates containing a high percentage of xylose. This strain carries pYES2-X123, a 2 μ -based *URA3* vector

carrying the *P. stipitis* *XYL1*, *XYL2*, and *XYL3* genes under the control of strong constitutive promoters (Ni et al., 2007). A drop test was used to determine growth of this strain on plates and no growth was observed on 6% xylose even after 5 days of incubation. Interestingly, some growth was noted on the 2% xylose plates after approximately 3 days of growth, indicating that the inhibition might be dependent on sugar concentration (data not shown).

3.2. Deletion of *PHO13* enables growth on xylose

Deletion of the *PHO13* gene restored growth on xylose in the CMB.JHV.XYL123 background. A drop test was performed on 2% and 6% xylose plates to compare the growth of CMB.JHV.XYL123.pho13a to that of CMB.JHV.XYL123 (*PHO13*). Growth was much stronger in the deletion strain than CMB.JHV.XYL123, with strong growth being noted on the 2% xylose plates after only 2 days. Slight growth of the deletion strain was noted on the 6% xylose plate after 2 days, and strong growth was noted after 3 days.

3.3. Deletion of *PHO13* does not affect growth on glucose

Aerobic batch cultivations of CEN.PK. 113-7D and the *pho13Δ* strain CMB.JHV.pho13b (see Table 1) were carried out on 20 g L⁻¹ glucose to determine what effect, if any, deletion of the *PHO13* gene had on growth on glucose. The growth, sugar consumption, ethanol production (and subsequent consumption), and carbon dioxide evolution profiles of these two strains did not differ significantly (Fig. 1). When the production rates and yields generated during these fermentations (Table 2) were examined, the only significant difference appeared to be in acetate production. CMB.JHV.pho13b had a specific rate of acetate production (0.32 Cmmol g DCW⁻¹ h⁻¹) that is 1.8 times greater than that of the wild type (0.18 Cmmol

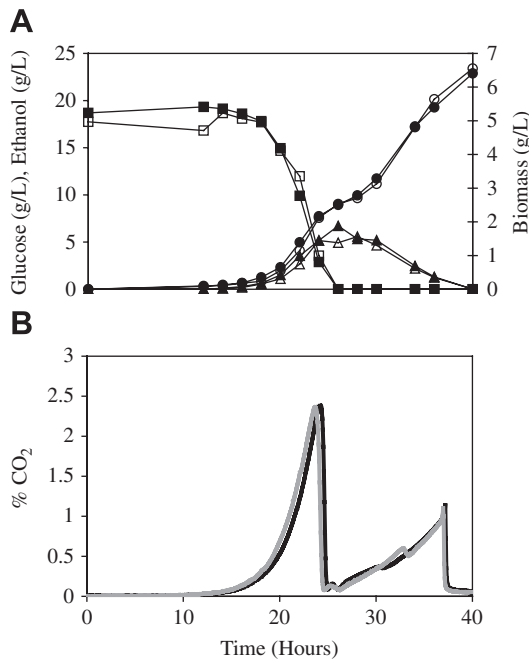


Fig. 1. Aerobic glucose cultivations: (A) glucose (■ and □), ethanol (▲ and △), and biomass (● and ○). Closed symbols, *S. cerevisiae* CEN.PK.113 7D; open symbols, CMB.JHV.pho13b. Part (A) shows the average of results from triplicate batch cultivations. (B) Carbon dioxide evolution profiles. Black line, CEN.PK.113 7D; gray line, CMB.JHV.pho13b.

Table 2
Rates and yields for aerobic glucose cultivations^a

Strain	$\mu_{\max}^b$	r_{glu}^c	r_{ETOH}^d	r_{gly}^e	r_{Ac}^f	$Y_{(x/s)}^g$	$Y_{(\text{ETOH}/s)}^h$	$Y_{(\text{gly}/s)}^i$	$Y_{(\text{Ac}/s)}^j$	$Y_{(\text{pyr}/s)}^k$
CEN.PK 113 7D	0.31 ± 0.01	21.97 ± 0.52	8.89 ± 1.20	1.14 ± 0.20	0.18 ± 0.09	0.15 ± 0.004	0.41 ± 0.06	0.05 ± 0.01	0.008 ± 0.004	0.001 ± 0.001
CMB.JHV.pho13b	0.32 ± 0.004	20.89 ± 1.9	9.21 ± 1.85	0.96 ± 0.36	0.32 ± 0.06	0.163 ± 0.014	0.44 ± 0.06	0.05 ± 0.02	0.015 ± 0.002	0.005 ± 0.0004

^aCultivations were performed in triplicate avg. ± std. dev.

^bSpecific growth rate (h⁻¹).

^cGlucose consumption rate (Cmmol g DCW⁻¹ h⁻¹).

^dEthanol production rate (Cmmol g DCW⁻¹ h⁻¹).

^eGlycerol production rate (Cmmol g DCW⁻¹ h⁻¹).

^fAcetate production rate (Cmmol g DCW⁻¹ h⁻¹).

^gBiomass yield (Cmmol/Cmmol).

^hEthanol yield (Cmmol/Cmmol).

ⁱGlycerol yield (Cmmol/Cmmol).

^jAcetate yield (Cmmol/Cmmol).

^kPyruvate yield (Cmmol/Cmmol).

$\text{gDCW}^{-1}\text{h}^{-1}$). The overall yield of acetate was also 1.9 times higher in the mutant strain (0.015Cmmol/Cmmol) than in the wild type strain (0.008Cmmol/Cmmol).

3.4. Deleting the *PHO13* gene increases xylose consumption in mixed sugar cultivations

Cultivation of CMB.JHV.XYL123 and CMB.JHV.XYL123.pho13a on 10g L^{-1} glucose and 10g L^{-1} xylose (Fig. 2) showed three distinct phases of growth. In the first phase glucose was consumed and ethanol was produced by both strains with little to no xylose consumption. In the second phase ethanol and xylose were consumed simultaneously, and in the third phase xylose and glycerol were consumed.

The specific growth rate on glucose and specific glucose uptake rate of these strains were comparable to each other, but they were notably lower than those of wild type CEN.PK.113-7D and the engineered CMB.JHV.pho13b grown on pure glucose. Once again, a higher acetate yield was seen in fermentations with the *PHO13* disruption (Table 3); however, in these cultivations we also observed a 1.3-fold lower glycerol yield for the mutant than from the wild type.

Many differences were noted between the two strains during xylose and ethanol co-consumption. The specific growth rate and rate of specific xylose uptake were approximately 1.5 times higher in the deletion strain (0.13h^{-1} and $2.68\text{Cmmol gDCW}^{-1}\text{h}^{-1}$, respectively) than in the parental strain (0.09h^{-1} and $1.69\text{Cmmol gDCW}^{-1}\text{h}^{-1}$). The volumetric xylose consumption rate of the deletion strain was more than double than that of the parental strain. The volumetric ethanol consumption rate

was also higher by 1.3-fold in the deletion strain; however, this increase was not observed when the specific consumption rates were compared.

During the final phase of these cultivations, xylose and glycerol were co-consumed. The rates and yields for the two strains were very similar during this phase, with the exception being the volumetric consumption rate for xylose (Table 3). Xylose concentrations in the *pho13Δ* cultivations have dropped below 5g L^{-1} by the beginning of this phase, while they remain significantly higher in the parental cultivations. This contributed to the lower specific rate of xylose uptake, as the glucose transporters in *S. cerevisiae* have a low affinity for xylose and at such low concentrations uptake is slowed.

3.5. Deletion of *PHO13* has a dramatic effect during growth on xylose

We observed a dramatic difference between these two strains when they were grown on xylose as a sole carbon source under fully aerobic conditions. Both the maximum specific growth rate and volumetric xylose consumption rate of the deletion strain, 0.10h^{-1} and 20.8Cmmol h^{-1} , respectively, were over 10 times greater than those observed in the parental strain cultivations (Table 4) (Fig. 3). The specific rate of xylose consumption was more than 1.5 times higher in the deletion strain than in the parental. Glycerol production, however, was reduced in the deletion strain with this being reflected in both the specific rate of glycerol production and the glycerol yield (Table 4) (Fig. 3).

A less dramatic, but still significant, difference in specific growth rate was observed between the two strains under

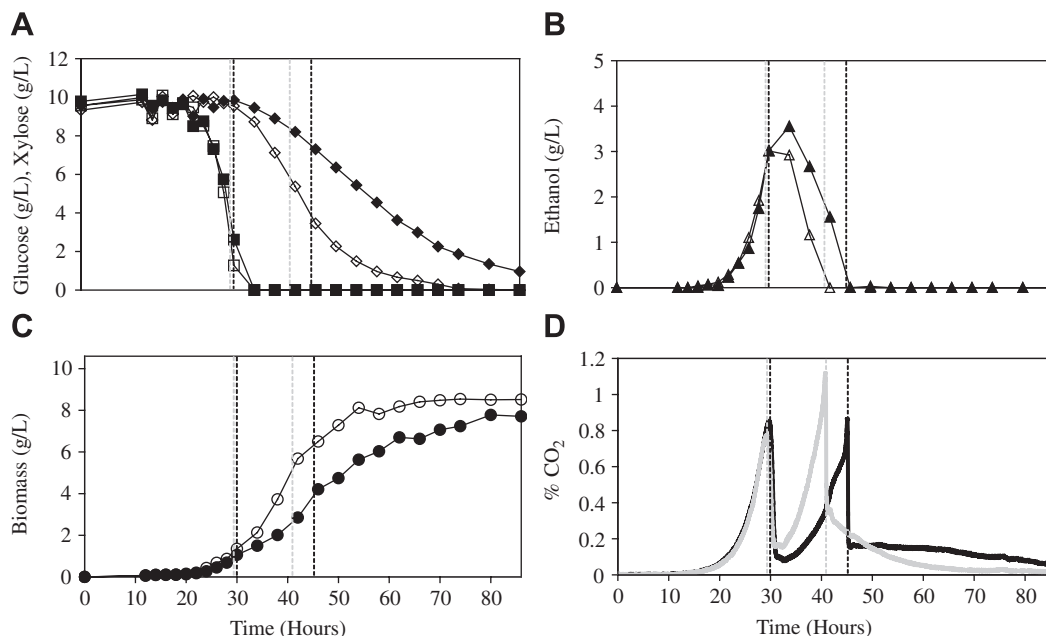


Fig. 2. Aerobic mixed sugar cultivations: closed symbols, *S. cerevisiae* CMB.JHV.XYL123; open symbols, CMB.JHV.XYL123.pho13a. (A) Glucose (■ and □) and xylose (◆ and ◇), (B) ethanol (▲ and △), (C) biomass (● and ○). Parts (A–C) show the average of results from triplicate batch cultivations. (D) Carbon dioxide evolution profiles. Black line, CMB.JHV.XYL123; gray line, CMB.JHV.XYL123.pho13a.

Table 3
Rates and yields for mixed sugar cultivations^a

Strain	Rates					Yields				
	$\mu_{\max}^b$	r_{glu}^c	r_{ETOH}^d	r_{gly}^e	r_{Ac}^f	$Y_{(x/s)}^g$	$Y_{(\text{ETOH}/s)}^h$	$Y_{(\text{gly}/s)}^i$	$Y_{(\text{Ac}/s)}^j$	$Y_{(\text{pyr}/s)}^k$
<i>Glucose consumption</i>										
CMB.JHV.XYL123	0.23 ± 0.06	13.79 ± 1.66	7.32 ± 0.74	0.59 ± 0.05	0.29 ± 0.08	0.17 ± 0.02	0.53 ± 0.04	0.05 ± 0.005	0.01 ± 0.004	0.01 ± 0.01
CMB.JHV.XYL123.pho13a	0.22 ± 0.03	12.40 ± 0.78	5.78 ± 1.61	0.29 ± 0.08	0.27 ± 0.11	0.18 ± 0.01	0.46 ± 0.01	0.038 ± 0.007	0.03 ± 0.01	0.01 ± 0.01
	Specific rates					Volumetric rates				Yields
	$\mu_{\max}^b$	r_{xyl}^l			r_{ETOH}^m	R_{xyl}^n		R_{ETOH}^o		$Y_{(x/s)}^g$
<i>Xylose–ethanol co-consumption</i>										
CMB.JHV.XYL123	0.09 ± 0.02		1.69 ± 0.09		2.67 ± 1.19	5.32 ± 0.14		8.18 ± 3.22		0.49 ± 0.02
CMB.JHV.XYL123.pho13a	0.13 ± 0.004		2.68 ± 0.22		2.53 ± 0.62	11.68 ± 0.39		10.89 ± 2.36		0.66 ± 0.09
	Rates					Yields				
	$\mu_{\max}^b$	r_{xyl}^l	r_{XOL}^p	r_{pyr}^q		R_{xyl}^n	$Y_{(x/s)}^g$	$Y_{(\text{XOL}/s)}^r$	$Y_{(\text{pyr}/s)}^k$	
<i>Xylose consumption</i>										
CMB.JHV.XYL123	0.03 ± 0.01	1.67 ± 0.25	0.005 ± 0.008	0.0003 ± 0.0005		7.21 ± 2.09	0.69 ± 0.11	0.002 ± 0.004	0.002 ± 0.004	
CMB.JHV.XYL123.pho13a	0.03 ± 0.01	1.71 ± 0.40	0.002 ± 0.002	0.003 ± 0.005		10.75 ± 0.80	0.64 ± 0.13	0.001 ± 0.002	0.002 ± 0.003	

^aCultivations were performed in triplicate avg. ± std. dev.

^bSpecific growth rate (h^{-1}).

^cGlucose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^dSpecific ethanol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^eSpecific glycerol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^fSpecific acetate production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^gBiomass yield (Cmmol/Cmmol).

^hEthanol yield (Cmmol/Cmmol).

ⁱGlycerol yield (Cmmol/Cmmol).

^jAcetate yield (Cmmol/Cmmol).

^kPyruvate yield (Cmmol/Cmmol).

^lSpecific xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^mSpecific ethanol consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

ⁿVolumetric xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^oVolumetric ethanol consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^pSpecific xylitol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^qSpecific pyruvate production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^rXylitol yield (Cmmol/Cmmol).

Table 4
Rates and yields from xylose cultivations^a

Strain	$\mu_{\max}^b$	r_{xyt}^c	r_{gly}^d	r_{XOL}^e	R_{xyt}^f	$Y_{(x/s)}^g$	$Y_{(\text{XOL}/s)}^h$	$Y_{(\text{gly}/s)}^i$
CMB.JHV.XYL123	0.01 ± 0.00	1.86 ± 0.58	0.59 ± 0.23	0.03 ± 0.03	1.86 ± 0.96	0.31 ± 0.09	0.02 ± 0.02	0.31 ± 0.07
CMB.JHV.XYL123.pho13a	0.10 ± 0.01	3.45 ± 0.13	0.035 ± 0.005	0.01 ± 0.01	20.76 ± 2.96	0.56 ± 0.11	0.002 ± 0.001	0.01 ± 0.003

^aCultivations were performed in triplicate avg. ± std. dev.

^bSpecific growth rate (h^{-1}).

^cMaximum specific xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^dGlycerol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^eXylitol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^fVolumetric xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^gBiomass yield (Cmmol/Cmmol).

^hXylitol yield (Cmmol/Cmmol).

ⁱGlycerol yield (Cmmol/Cmmol).

Table 5
Rates and yields from low oxygen xylose cultivations^a

Strain	Specific rates					Volumetric rates	
	$\mu_{\max}^b$	r_{xyt}^c	r_{EtOH}^d	r_{XOL}^e	r_{gly}^f	R_{xyt}^g	R_{EtOH}^h
CMB.JHV.XYL123	0.017 ± 0.01	4.96 ± 0.94	0.07 ± 0.01	0.026 ± 0.018	0.31 ± 0.13	2.97 ± 1.26	0.09 ± 0.01
CMB.JHV.XYL123.pho13a	0.062 ± 0.00	4.85 ± 0.44	0.64 ± 0.03	0.019 ± 0.005	0.18 ± 0.02	15.47 ± 0.72	2.05 ± 0.19
	Yields						
	$Y_{(x/s)}^i$	$Y_{(x/c)}^j$	$Y_{(x/s)}^k$	$Y_{(\text{XOL}/s)}^l$	$Y_{(\text{gly}/s)}^m$	Y_{EtOH}^n	
CMB.JHV.XYL123	0.42 ± 0.08	0.32 ± 0.02	0.35 ± 0.01	0.007 ± 0.005	0.149 ± 0.076	0.066 ± 0.015	
CMB.JHV.XYL123.pho13a	0.38 ± 0.03	0.45 ± 0.01	0.45 ± 0.02	0.006 ± 0.001	0.037 ± 0.008	0.315 ± 0.014	

^aCultivations were performed in duplicate avg. ± std. dev.

^bMaximum specific growth rate (h^{-1}).

^cMaximum specific xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^dEthanol production rate.

^eXylitol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^fSpecific glycerol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^gVolumetric xylose consumption rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

^hVolumetric ethanol production rate ($\text{Cmmol g DCW}^{-1} \text{h}^{-1}$).

ⁱBiomass yield (Cmmol/Cmmol) 12–32 h based on xylose consumed.

^jBiomass yield (Cmmol/Cmmol) 22–72 h based on total carbohydrates.

^kBiomass yield (Cmmol/Cmmol) 12–72 h based on xylose consumed.

^lXylitol yield (Cmmol/Cmmol).

^mGlycerol yield (Cmmol/Cmmol).

ⁿEthanol yield (Cmmol/Cmmol).

low oxygen conditions. The maximum specific growth rate was 3.5 times higher in the deletion strain than in the parental strain (Table 5) (Fig. 4). While the specific rates of xylose consumption were very similar between the two strains, due to the increased specific growth rate, the volumetric xylose consumption rate continued to be higher in the deletion strain. Glycerol production also continued to be lower in this strain than in the parental.

We observed ethanol production in both strains, but ethanol production was dramatically higher in the deletion strain. The *pho13Δ* strain showed a specific ethanol production rate 10-fold greater than that of its parent. Combined with the increased specific growth rate, the overall ethanol production rate is over 20 times greater in the deletion strain. Ethanol yield based on the xylose

consumed is fivefold greater in the deletion strain than in its parent (Table 5) (Fig. 4).

4. Discussion

Relatively little is known about the physiological function of Pho13, but our findings confirm the observations by Ni et al. (2007) that deletion of *PHO13* improves the utilization of xylose in yeast. *PHO13* has been reported to possess *p*-nitrophenyl phosphatase activity against phosphorylated proteins, particularly histones. Similar proteins are widely distributed in other yeasts and fungi, where it consists essentially of a single copy in each genome (Fig. 5). The exact mechanism by which deletion of the

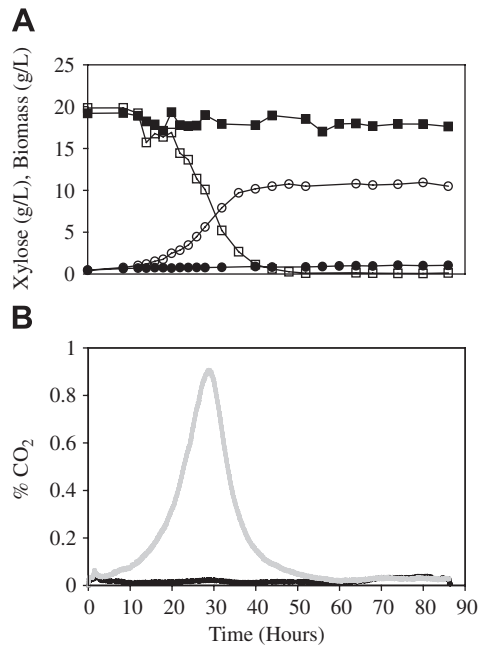


Fig. 3. Aerobic xylose cultivations: (A) xylose (■ and □) and biomass (● and ○). Closed symbols, *S. cerevisiae* CMB.JHV.XYL123; open symbols, CMB.JHV.XYL123.pho13a. Part (A) shows the average of results from triplicate batch cultivations, (B) carbon dioxide evolution profiles. Black line, CMB.JHV.XYL123; gray line, CMB.JHV.XYL123.pho13a.

PHO13 gene improves growth and ethanol production on xylose, however, is still to be elucidated.

We observed lower specific growth rates of strains carrying plasmids as compared to the equivalent strains without vectors. The specific glucose uptake rates were also lower. This is likely due to plasmid burden, as a lower specific growth rate is also observed when growing these strains on pure glucose (data not shown), with some contribution of competition of glucose and xylose for the glucose transporters to the lowered specific uptake rate.

The increase in acetate production and decrease in glycerol production observed in the *pho13Δ* strain may indicate an altered redox state. The 1.8-fold higher rate of acetate production observed in the *pho13Δ* mutant grown on glucose could result from higher levels of acetaldehyde dehydrogenase or pyruvate decarboxylase and therefore a higher rate of NAD(P)⁺ reduction. The 10-fold decrease in glycerol accumulation observed in the *pho13Δ* strain grown on xylose could be a result of higher levels of glycerol kinase converting glycerol to glycerol-3-phosphate. Glycerol-3-phosphate dehydrogenase and triosephosphate isomerase would then be necessary to convert the glycerol-3-phosphate into glyceraldehyde-3-phosphate which could then enter either the glycolytic pathway or the gluconeogenesis pathway. Another, more likely explanation would be a more balanced redox situation in the *pho13* deletion strain. Since glycerol production is a pathway commonly used by *S. cerevisiae* to reoxidize NADH (Bakker et al., 2001), lower glycerol production, as seen in CMB.JHV.

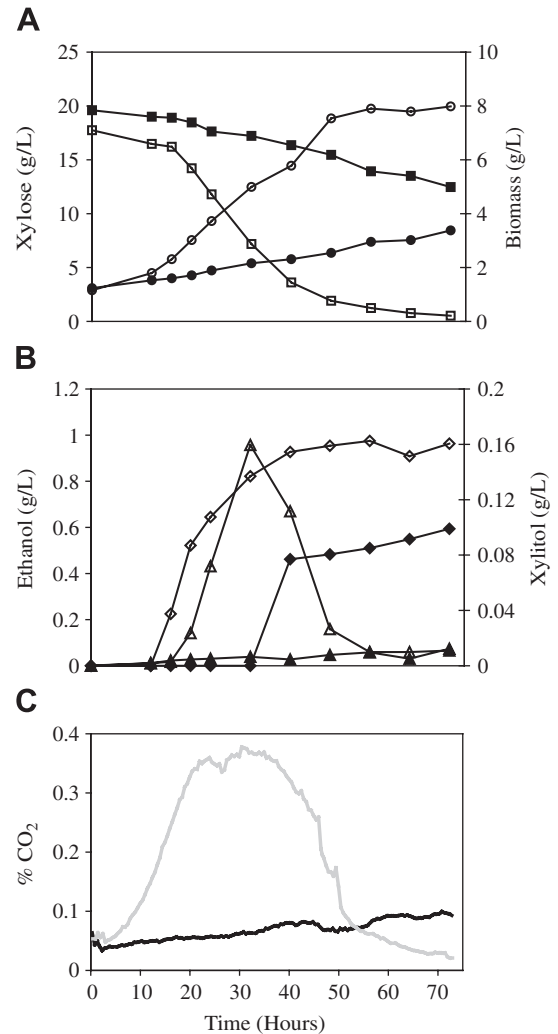


Fig. 4. Low oxygen xylose cultivations: closed symbols, *S. cerevisiae* CMB.JHV.XYL123; open symbols, CMB.JHV.XYL123.pho13a. Results shown are the average of duplicates. (A) Xylose (■ and □) and biomass (● and ○), (B) ethanol (▲ and △) and xylitol (◆ and ◇), (C) carbon dioxide evolution profiles. Black line, CMB.JHV.XYL123; gray line, CMB.JHV.XYL123.pho13a.

XYL123.pho13a, could indicate less excess NADH produced during assimilation and metabolite production.

This is the first report of xylose inhibition of engineered *S. cerevisiae* as a function of the xylose concentration. Six percent xylose completely inhibited growth of CMB.JHV.XYL123 on agar plates, whereas 2% xylose did not. In contrast, neither concentration of xylose blocked growth of the *pho13Δ* mutant. This differential inhibition might be attributed to an osmotic effect, but it could also be due to an increased xylose uptake rate at the higher sugar concentration. In either case it could also help explain some of the varying results reported by different laboratories. We know that *TAL1* up-regulation mutations can relieve growth inhibition and that these are readily selected for when cells expressing high levels of xylulokinase (XYL3) activity are cultivated on mixtures of glucose and xylose (Pitkänen et al., 2005, Ni et al., 2007). A similar

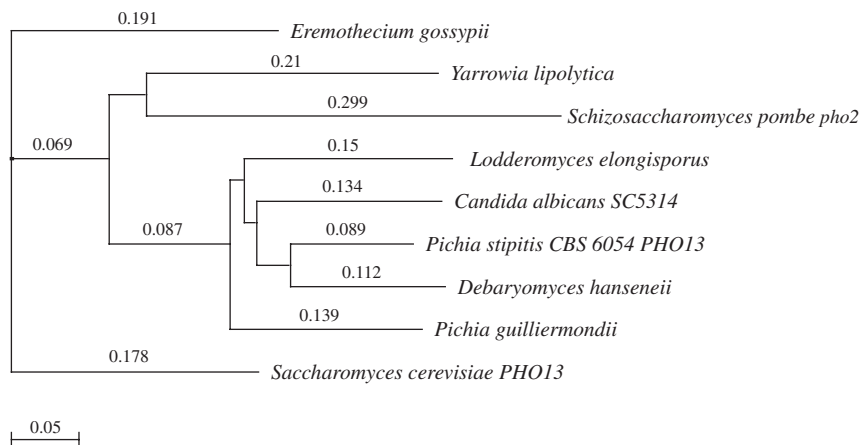


Fig. 5. Phylogenetic relatedness of *Saccharomyces cerevisiae* PHO13 with similar proteins from other fungi. The tree was constructed by neighbor joining, best tree with systematic tie breaking, and gaps were distributed proportionally.

selection might occur on plates of 2% xylose or in liquid medium once glucose is exhausted.

The relationship between growth inhibition and oxygen availability is complex. The parental strain CMB.JHV.XYL 123 is subject to growth inhibition resulting from high xylulose flux to *PsXYL3*. The higher specific growth rate of this strain in microaerobic culture as opposed to aerobic culture is most likely due to lower toxicity resulting from decreased flux under low aeration conditions. Work by Ni et al. (2007) showed that when xylulokinase was overexpressed, growth was almost completely inhibited on agar plates, where air can easily diffuse into the medium. In liquid broth, however, where oxygen levels are somewhat lower, growth occurred slowly. Lower aeration under microaerobic conditions, can therefore explain the increased growth seen here in the parental strain.

S. cerevisiae engineered for xylose metabolism with the oxidoreductase pathway will grow on xylose only if oxygen is available. Work by Toivari et al. (2001) showed that as the aeration level decreased, the levels of xylulose-5-phosphate accumulating in the cell increased. At the same time, as the aeration level increased, the ATP levels detected in the cells decreased. These results indicate that as the aeration level increases, so does the rate of metabolism downstream of xylulose-5-phosphate. These findings are consistent with a greater use of intermediary metabolites and ATP for cell growth in the presence of oxygen.

In conclusion, we have shown that deletion of the *PHO13* gene dramatically improves growth on xylose as a sole carbon source when *XYL3* is overexpressed. Ethanol production is also considerably increased when the strain is grown under low oxygen conditions. A change in redox balance within the cell is suggested by an observed increase in acetate production and decrease in glycerol production by the *pho13* deletion strain. These changes could be attributable to regulatory effects of the phosphatase. Because several studies (Jin and Jeffries, 2003; Jin et al.,

2002, 2005) have indicated that xylulokinase levels are potentially a limiting step in xylose metabolism, development of strains expressing a high level of this enzyme may dramatically improve bioethanol production from lignocellulosic feedstocks. Growth inhibition due to *XYL3* overexpression, which has been reported previously (Jin et al., 2003; Johansson et al., 2001; Ni et al., 2007) and confirmed in this study, however, is a hurdle that must be overcome in development of these strains. The presented research provides compelling evidence that deletion of the *PHO13* gene is one such method to overcome growth inhibition due to overexpression of xylulokinase.

Acknowledgments

This research was supported by a Fulbright grant from the Danish American Fulbright Commission to J.H.V.V. This work was supported by the Danish Research Agency and by US NIH Grant 5R01 GM67933-03 to T.W.J. We would like to thank Goutham Vemuri and Jose Manuel Otero for help with rate and yield calculations and Christian Solem for help with HPLC.

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