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ORIGINAL PAPER

Engineering industrial *Saccharomyces cerevisiae* strains for xylose fermentation and comparison for switchgrass conversion

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Abstract *Saccharomyces*' physiology and fermentation-related properties vary broadly among industrial strains used to ferment glucose. How genetic background affects xylose metabolism in recombinant *Saccharomyces* strains has not been adequately explored. In this study, six industrial strains of varied genetic background were engineered to ferment xylose by stable integration of the xylose reductase, xylitol dehydrogenase, and xylulokinase genes. Aerobic growth rates on xylose were 0.04–0.17 h^{-1}. Fermentation of xylose and glucose/xylose mixtures also showed a wide range of performance between strains. During xylose fermentation, xylose consumption rates were 0.17–0.31 g/l/h, with ethanol yields 0.18–0.27 g/g. Yields of ethanol and the metabolite xylitol were positively correlated, indicating that all of the strains had downstream limitations to xylose metabolism. The better-performing engineered and parental strains were compared for conversion of alkaline pretreated switchgrass to ethanol. The engineered strains produced 13–17% more ethanol than the parental control strains because of their ability to ferment xylose.

Keywords Xylose · Bioethanol · Switchgrass · *Saccharomyces* · Industrial yeast

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Introduction

Intense interest in achieving energy security and reducing carbon emissions has led to a global research effort towards developing renewable sources of liquid transportation fuels. Presently, the United States (2009) produces 10.6 billion gallons (40.1 billion liters) of ethanol per year, displacing 364 million barrels of oil [25]. Global production has also been increasing and has now reached 19.5 billion gallons (73.8 billion liters) per year, the majority (88%) originating from the US and Brazil. Most of the ethanol produced in the US is from fermentation of corn starch. Expanding biofuel production further in the US will largely depend upon developing lignocellulosic materials as feedstocks. Sources of lignocellulose include agriculture resides (e.g., corn stover), wood processing side-streams, and dedicated energy crops. While glucose is the most abundant sugar in these feedstocks, 20–40% of lignocellulosic biomass is hemicellulose, which consists mostly of xylose [26]. Therefore, fermentation of both glucose and xylose is necessary for cellulosic ethanol to be commercially feasible [10, 12, 20]. For the US, the Renewable Fuels Standard targets production of 16 billion gallons of advanced biofuels from lignocellulose by 2022. Achieving this ambitious target will require developing improved conversion processes and especially new microbial biocatalysts.

Saccharomyces yeasts have been engineered to ferment xylose by either a coupled reduction/oxidation or an isomerization reaction. Both pathways convert xylose to xylulose, which is phosphorylated to xylulose-5-phosphate, which then enters the pentose-phosphate pathway (PPP). Significant progress has been made using both strategies [5, 11, 31]. Much of this work has been done using laboratory strains due to their ease of genetic manipulation, but

unlike industrial strains, they have not been selected for producing ethanol on a large scale. For commercial-scale conversion of biomass-derived sugars to ethanol (and other renewable products), industrial strains will be required.

Saccharomyces yeasts possess many properties that make them highly desirable for use in industrial lignocellulose fermentation processes (reviewed in [5, 9, 10, 19, 20]). These properties include tolerance to high ethanol concentrations, fermentation at low pH (to minimize bacterial contamination), high ethanol productivity and yield from glucose, and tolerance to fermentation inhibitors. Compared to the available literature using laboratory strains, relatively few industrial yeasts have been transformed with the required genes for xylose-fermentation [10, 21, 29]. Additionally, most prior studies have focused on a single genetic background and therefore do not fully exploit the genetic diversity available within yeast collections. The objectives of this study were to better understand the influence of genetic background on xylose fermentation in recombinant industrial *Saccharomyces* strains and to identify promising strains for further development.

Six *Saccharomyces* strains of varied genetic background were engineered to ferment xylose by stable chromosomal integration of the xylose reductase, xylitol dehydrogenase, and xylulokinase genes. All six strains have either been isolated from distillery-type operations or otherwise recommended for lignocellulose conversion. The engineered strains were compared for their growth and fermentation rates of xylose and xylose/glucose mixtures in aerobic and anaerobic cultures. Finally, the best xylose-fermenting strains were evaluated for ethanol production using ammonium hydroxide pretreated switchgrass hydrolysate in a simultaneous saccharification and fermentation (SSF).

Materials and methods

Strains, media, and general methods

Escherichia coli strains DH10B, TOP10 (Invitrogen, Carlsbad, CA, USA), NEB5a, and NEB10b (New England Biolabs (NEB), Ipswich, MA, USA) were used for routine maintenance and preparation of plasmids and were grown in LB medium [27]. Yeast strains and plasmids used in this study are listed in Table 1. DNA was transformed into yeast cells using a standard lithium acetate method [8]. Synthetic complete (SC) medium consisted of 6.7 g/l Difco yeast nitrogen base (YNB) (United States Biological, Marblehead, MA, USA), and was supplemented with amino acids as necessary [1]. YP medium (10 g/l yeast extract, 20 g/l bacto-peptone) was autoclaved without carbohydrate. Sterile glucose or xylose was added separately.

Generation of plasmids for integrating the *XYL1*/*XYL2*/*XKS1* expression cassette

Yeast expression vectors were generated by PCR amplification of the *HXT7*, *PGK1*, and *ADH1* promoters using primer pairs 19/20, 23/24, and 27/28, respectively. The *HXT7*, *PGK1*, and *ADH1* terminators were separately amplified using primer pairs 21/22, 25/26, and 29/30. DNA fragments for cloning were amplified using *PfuTurbo* Hotstart polymerase (Stratagene, La Jolla, CA, USA). Adenosine overhangs were added with *Taq* polymerase (NEB) and each fragment was cloned into pCR2.1-topo (Invitrogen) and sequenced. Error-free promoter and terminator fragments were sub-cloned into vectors pRS413, pRS414 and pRS416 [4] using restriction enzyme sites that were incorporated into the primer sequence. DNA oligomers used in this study are listed in Table 2.

The *XYL1* and *XYL2* genes were PCR amplified using genomic DNA from *Pichia stipitis* (Y-7124, obtained from the USDA-ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA) using primer pairs 31/32 and 33/34. The *Saccharomyces cerevisiae XKS1* gene was PCR amplified from genomic DNA (BY4700) using primers 61 and 62. All genes were first cloned into pCR2.1-topo for sequencing. Sequence-verified fragments containing the genes were sub-cloned into yeast expression vectors. The *XYL1* gene was digested with *Spe*I and *Cla*I and cloned into pRH152 (containing P_{PGK1}-MCS-T_{PGK1}) to create pRH185. The *XYL2* gene was digested with *Spe*I and *Sal*I and cloned into pRH133 (containing P_{ADH1}-MCS-T_{ADH1}) to create pRH169. The *XKS1* gene was digested with *Spe*I and *Sal*I and cloned into pRH164 (containing P_{HXT7}-MCS-T_{HXT7}) to create pRH195. The *Xho*I-*Sac*I fragment from pRH185, containing P_{ADH1}-*XYL2*-T_{ADH1}, was blunt-ended with T4 DNA polymerase (NEB) and cloned into the blunt-ended and dephosphorylated *Sac*I site of the *XYL1* expression vector (pRH185) to create pRH211, which expresses both *XYL1* and *XYL2*. To create a vector expressing the xylulokinase as well, the *Bss*HII fragment from pRH195, containing P_{HXT7}-*XKS1*-T_{HXT7}, was blunt-ended and cloned into the blunt, and dephosphorylated, *Xho*I site of pRH211, creating pRH275. To generate the vector pRH277, used to target the *XYL1*/*XYL2*/*XKS1* expression cassette for chromosomal integration at the *HO* locus of *S. cerevisiae*, the *Bss*HII fragment containing the *XYL1*/*XYL2*/*XKS1* cassette was cloned into the dephosphorylated *Bss*HII site in the polylinker of the vector *HO*-KanMX4-poly-*HO* [32]. To generate industrial strains with the chromosomally integrated *XYL1*/*XYL2*/*XKS1* cassette, pRH277 was digested with *Not*I to liberate the KanMX4-*XYL1*/*XYL2*/*XKS1* with flanking homology to target the genes to the *HO* endonuclease locus on chromosome IV (Fig. 1). The *HO*-KanMX4-*XYL1*/*XYL2*/*XKS1*-*HO*

Table 1 Yeast strains and plasmids used in this study

Strain	Genotype	Reference
Y-7124	*Pichia stipitis*	ARS[a]
CEN.PK2-1C	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3*Δ*1 MAL2-8*^{C} *SUC2*	Euroscarf
D5A	*S. cerevisiae* isolated from cheese whey	ATCC
Y-1528	*S. cerevisiae*, natural galactose-assimilating isolate	ARS
Y-1649	*S. cerevisiae* isolated from wood hydrolysates	ARS
Y-2034	*S. cerevisiae* isolated from wine	ARS
Y-7494	*S. cerevisiae* isolated from a molasses distillery	ARS
Y-11780	*S. cerevisiae* isolated from refrigerated bourbon wort	ARS
YB-1778	*S. cerevisiae* isolated from fermented cassava mash	ARS
YB-2625	*S. cerevisiae* isolated from Bagasse	ARS
YRH388	CEN.PK2-1C *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH390	Y-2034 *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH392	Y-7494 *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH394	YB-1778 *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH396	YB-2625 *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH400	D5A*?::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
YRH403	Y-1528 *ho*Δ*::*(KanMX4; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	This work
Plasmid	**Description**	**References**
pRS413	pBluescript II SK+, *HIS3, CEN6, ARSH4*	[4]
pRS414	pBluescript II SK+, *TRP1, CEN6, ARSH4*	[4]
pRS416	pBluescript II SK+, *URA3, CEN6, ARSH4*	[4]
HO-poly-KanMX4-*HO*	Vector for targeted integration at the *HO* locus	[32]
pRH133	pRS413 + P_{ADH1}-MCS-T_{ADH1}	This work
pRH152	pRS416 + P_{PGK1}-MCS-T_{PGK1}	This work
pRH164	pRS414 + P_{HXT7}-MCS-T_{HXT7}	This work
pRH169	pRS413 + P_{ADH1}-*XYL2*-T_{ADH1}	This work
pRH185	pRS416 + P_{PGK1}-*XYL1*-T_{PGK1}	This work
pRH195	pRS414 + P_{HXT7}-*XKS1*-T_{HXT7}	This work
pRH211	pRS416 + P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}	This work
pRH275	pRS416 + P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7}	This work
pRH277	*HO*-poly-KanMX4-*HO* + P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7}	This work

[a] Stains were obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL

DNA was transformed into yeast cells using a standard lithium acetate method [8]. Cells were allowed to recover in YPD for 4 h prior to plating to YPD + 200 μg/ml G418 plates. Integration of the KanMX4-*XYL1*/*XYL2*/*XKS1* was confirmed by PCR using primers 31 and 32 to amplify the *XYL1*gene. Chromosomal integration at the *HO* locus was confirmed using a primer with homology to the KanMX4 gene of the integrating fragment (#235) and a reverse primer with homology to the chromosome flanking the integration site (#236). The strains were also confirmed to be heterozygous for the integration by PCR verification of the presence of wild-type *HO* sequence using primers 251/252. For strain YRH400, integration of the KanMX4-*XYL1*/*XYL2*/*XKS1* cassette was confirmed by the amplification of the *XYL1* gene. However, integration at the *HO* locus in YRH400 could not be confirmed.

Aerobic growth determination

Pre-cultures were grown to exponential phase in YPD and washed with sterile water prior to inoculation. Synthetic complete medium + 50 g/l xylose (SC5X) was used to determine each strain's ability to assimilate xylose aerobically. SC5X cultures were started at an OD_{660} of 0.05 and

Table 2 DNA oligos used in this study

RHFBT#	Sequence
19	5′-TAG*GAGCTC*TTTCGGGCCCCTGC-3′
20	5′-AGCGTCTTGTG*ACTAGT*TTTGATT-3′
21	5′-G*GTCGAC*GCGAACACTTTTATTAATTC-3′
22	5′-G*CTCGAG*TATTTGTGAATAACAGTGCGGTC-3′
23	5′-G*GAGCTC*ACCCTCATACTATTATCAG-3′
24	5′-GAAGATAA*ACTAGT*TGTTTTATATTTGTTG-3′
25	5′-G*GTCGAC*TAAATTGAATTGAATTGAAATCGA-3′
26	5′-G*CTCGAG*TAACGAACGCAGAATTTTCGAG-3′
27	5′-G*GAGCTC*CGGGTGTACAATATGGA-3′
28	5′-GG*ACTAGT*GTATATGAGATAGTTGATTGT-3′
29	5′-G*GTCGAC*TAAATAAGCGAATTTCTTATGAT-3′
30	5′-G*CTCGAG*CGACCTCATGCTATACCTGA-3′
31	5′-G*ACTAGT*GC**ATG**CCTTCTATTAAGTTGAACT-3′
32	5′-CTGCAGC*ATCGAT*TCTTAGACGAAGATAGGAATCT-3′
33	5′-C*ACTAGT*AA**ATG**ACTGCTAACCCTTCCTTG-3′
34	5′-G*GTCGAC*TTACTCAGGGCCGTCAATGA-3′
61	5′-G*ACTAGT***ATG**TTGTGTTCAGTAATTCAGA-3′
62	5′-G*GTCGAC*TTAGATGAGAGTCTTTTCCAGT-3′
235	5′-AGAGAGTTGTCACCAAGGCCAT-3′
236	5′-TATGGAACTGCCTCGGTGAG-3′
251	5′-TCTGAAAACACGACTATTCTGATGG-3′
252	5′-CTCAATTGAGCATCAAGATAATCCA-3′

Restriction endonuclease sites are shown *italicized* and *underlined*
Start codons are shown in *bold*

incubated at 30°C, shaking at 200 rpm. Specific growth rates were determined by linear regression of the $\ln(OD_{660})$ versus time with the growth rate μ (h^{-1}) as the slope. Experiments were repeated three times using independent cultures.

Batch fermentation

YP cultures (100 ml) with 50 g/l xylose (or 50 g/l xylose + 50 g/l glucose) were inoculated with exponentially growing cells to an OD_{660} of 2.0. CO_2 production was monitored continuously using a wireless gas production measurement system (Ankom Technologies; Macedon, NY, USA). The wireless system monitors gas production indirectly by measuring cumulative gas pressure; CO_2 production is calculated using the ideal gas law. The system was set to vent when the overhead pressure achieved 1 psi and to monitor the accumulated pressure every 15 min. At 96 h, samples were taken to determine cell biomass (by OD_{660}) and analyze remaining sugars and products formed (by high-performance liquid chromatography, HPLC). All experiments were performed three to four times. Accumulative pressure values were used to calculate the amount of CO_2 produced during the fermentation. These values were not used to calculate carbon recovery. Carbon recovery calculations were based on total input carbon from glucose and/or xylose and measured (HPLC) remaining glucose and xylose, along with fermentation products. CO_2 amounts used for carbon recovery calculations assumed 1 mol of CO_2 is produced for every 1 mol of ethanol.

Preparation and fermentation of aqueous ammonia pretreated switchgrass

The switchgrass used for this study was a lowland variety harvested post-frost, dried at 50°C, and ground in a cutting mill to pass through a 2-mm mesh. Ammonia-pretreated switchgrass (AM-PT-SG) was prepared by reacting switchgrass (SG) at 15% w/w solids, with 4.0% w/v ammonium hydroxide at 170°C for 20 min. The reaction was conducted using a 35-l working volume steam jacketed horizontal planetary mixer (custom fabricated Batch Ploughshare Horizontal Mixer, Littleford Day, Florence, KY, USA). Ammonia was evaporated by drying the whole hydrolysate at 50°C for 24 h. The resulting pretreated biomass was stored at ambient temperature and aliquots used for fermentation were sterilized by heating dry in an autoclave for 10 min. Fermentations contained 5 g of AM-PT-SG in 50 ml final volume (10% w/v) supplemented with GC220 cellulase (30 FPU/g SG), Novo188 β-glucosidase (40 U/g SG), Multifect pectinase (50 U/g SG), and YP. Surfactant (Pluronic F68) was included at 0.0186 g/ml because it improves enzymatic saccharification (data not shown). Culture pH was maintained by buffering at pH 4.5 with 50 mM citric acid. Fermentations were inoculated with

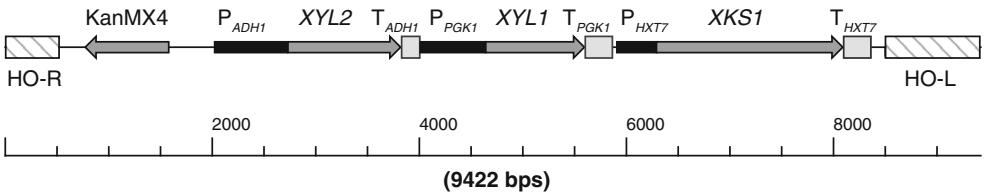

Fig. 1 Xylose utilization gene integration fragment. The integrating vector pRH277 contains the *P. stipitis XYL1* gene fused to the *PGK1* promoter, the *P. stipitis XYL2* gene fused to the *ADH1* promoter, and the *S. cerevisiae XKS1* gene fused to the *HXT7* promoter. The expression cassette shown is flanked by homology (HO-R and HO-L regions) to the *S. cerevisiae HO* endonuclease gene, which targets integration to the *HO* locus on chromosome IV

exponentially growing cells at a starting OD_{660} of 1.0 and incubated at 35°C for 96 h. The rate of ethanol fermentation was followed throughout by measuring CO_2 production using the Ankom wireless gas production system. At 96 h, samples were analyzed for residual sugars and fermentation products. AM-PT-SG fermentation experiments were replicated three times.

Analytical methods

Extracellular metabolites were measured using HPLC. Samples were analyzed using a SpectraSYSTEM liquid chromatography system (Thermo Electron Corporation, CA, USA) equipped with an automatic sampler, column heater, isocratic pump, refractive index detector, and computer-based integrator running Chromquest ver. 2.5 software (Thermo Electron Corporation). Samples were injected (20 µl) onto a sugar column (Aminex HPX-87H Column, 300 × 7.8 mm, Bio-Rad Laboratories, Inc.) and eluted with 5 mM sulfuric acid at 0.6 ml/min and 65°C.

Statistical analyses

For experiments with three or greater biological replicates, probability analyses were performed using Student's *t* test with a two-tailed distribution and compared to the appropriate control strain. Values with $p < 0.05$ were considered significant for this study. Statistical analysis was performed using Microsoft Excel.

Results

Chromosomal integration and xylose metabolism

Eight *Saccharomyces* yeast strains were chosen that were isolated from distillery operations or otherwise recommended for lignocellulosic fermentations (Table 1) [6, 7, 15, 16, 18, 23, 24, 35]. Additionally, the haploid laboratory strain, CEN.PK2-1C was included as a control. Each strain was engineered for xylose fermentation by chromosomal integration of the *Pichia stipitis* xylose reductase (*XYL1*), *P. stipitis* xylitol dehydrogenase (*XYL2*), and *S. cerevisiae* xylulokinase (*XKS1*) genes. Integration of the xylose utilization genes was successful for six of the eight industrial strains and the laboratory strain. The expression cassette, containing the *XYL1*, *XYL2*, and *XKS1* genes (Fig. 1), contains extended homology to chromosomal regions flanking the *HO* gene on chromosome IV, targeting integration to the *HO* locus. This region was chosen for several reasons. First, the HO endonuclease is only active in haploids and is not expected to be active in strains of higher ploidy. Second, it has been shown that deletion of the *HO* endonuclease gene does not affect growth rate or fermentation characteristics of brewing or wine yeasts [2, 34]. We compared the engineered strains to the parent strains for fermentation of 20% w/w glucose and found no significant differences in fermentation rate, yield, or ethanol tolerance (data not shown). Finally, the strains chosen for this study are diploid and can form four-spore asci. Deleting the *HO* gene in these strains makes it possible to generate a xylose-fermenting haploid with stable mating-type, making them amendable for classical genetic manipulations.

Table 3 Growth rate during aerobic growth on xylose

Strain	Parent strain	Growth rate μ (h^{-1})
YRH388	CEN.PK 2-1C	0.077 ± 0.006
YRH390	Y-2034	0.082 ± 0.010
YRH392	Y-7494	0.099 ± 0.006
YRH394	YB-1778	0.059 ± 0.004
YRH396	YB-2625	0.167 ± 0.004
YRH400	D5A	0.042 ± 0.003
YRH403	Y-1528	0.096 ± 0.005

Data shown represent the mean growth rate ± SD determined from three independent cultures grown in SC medium supplemented with 50 g/l xylose

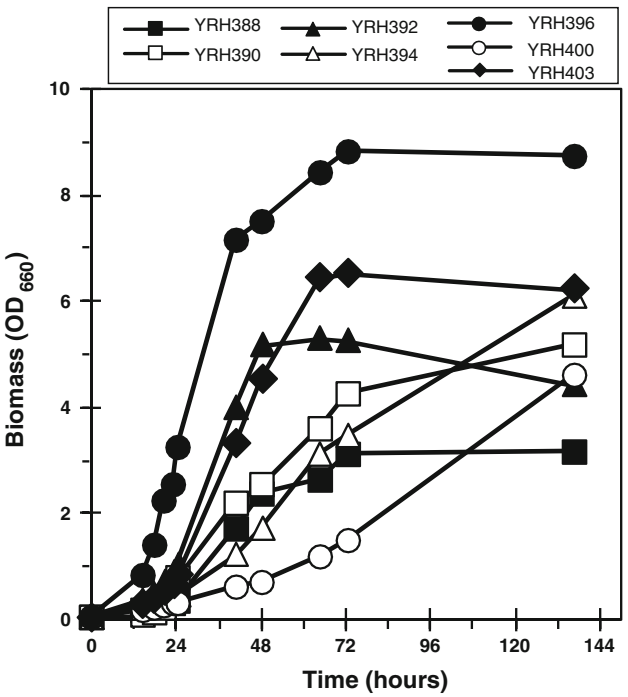

Fig. 2 Aerobic growth on xylose. Comparison of strains engineered to express the *XYL1*, *XYL2*, and *XKS1* genes. Strains were cultured in synthetic medium with 50 g/l xylose. Data shown are from a single representative growth analysis from experiments performed in triplicate

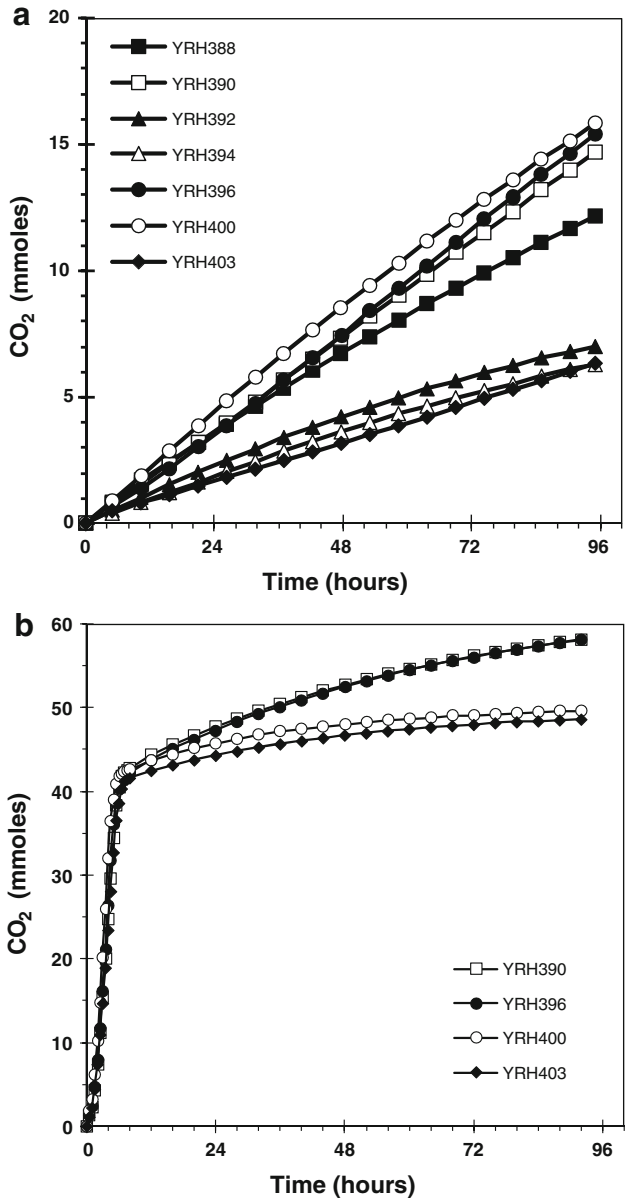

Fig. 3 Fermentation of xylose or a glucose/xylose mixture. Recombinant *Saccharomyces* strains engineered to ferment xylose were cultured under anaerobic conditions in YP medium containing **a** YP + xylose (50 g/l) or **b** YP + glucose (50 g/l) and xylose (50 g/l). CO_2 production was monitored continuously. Data shown represent the mean CO_2 production from three to four independent cultures

The ability of the engineered strains to assimilate xylose was confirmed by culturing them on synthetic complete medium with 50 g/l xylose (SC5X). None of the parent strains were able to grow on SC5X medium (data not shown). All of the engineered strains grew aerobically on xylose, indicating that the integrated genes were functionally expressed. However, there was a wide range of growth rates (0.042–0.167 h^{-1}) (Table 3) and final biomass concentrations (Fig. 2). YRH396 generated the most biomass and grew the fastest (Table 3), but in general, growth rate was not a predictor of final biomass formation.

The strains were next evaluated for their ability to ferment xylose to ethanol. Fermentation rates were monitored by measuring CO_2 production (Fig. 3a) and the cultures were analyzed for fermentation products at 96 h (Table 4). Three of the engineered industrial strains (YRH390, YRH396, and YRH400) produced up to 39% ($p = 0.02$) more ethanol and fermented xylose faster than the laboratory strain, and three strains (YRH392, YRH394, and YRH403) were slower and produced less ethanol ($p = 0.03$). The superior xylose-fermenting strains were further evaluated for fermentation of glucose/xylose mixtures.

Mixed substrate fermentation

From the xylose fermentation studies, YRH390, YRH396, YRH400, and YRH403 were selected for further study. The first three strains fermented xylose better than the laboratory strain. Strain YRH403 was included based on its reported unique fermentation properties and enhanced tolerance to inhibitors [14–16]. Since lignocellulosic hydrolysates are a mixture of primarily glucose and xylose, these strains were evaluated for their ability to ferment a 100 g/l equal mixture of both sugars. Fermentation rate and product formation were measured for each culture (Fig. 3b, Table 5). The strains produced comparable amounts of ethanol (28.1–32.9 g/l). Notably, strains YRH390 and YRH396 consumed more xylose than the other strains. However, the extra xylose consumed for YRH390 and YRH396 was not directed toward ethanol production, indicating metabolic limitations downstream of the xylose reductase and xylitol dehydrogenase.

Simultaneous saccharification and fermentation of ammonia-pretreated switchgrass hydrolysate

Strains YRH390, YRH396, and YRH400 were further evaluated for conversion of ammonia pretreated switchgrass (AM-PT-SG) to ethanol using a simultaneous saccharification and fermentation (SSF) strategy. The parent strains were included in this experiment to determine if increased ethanol productivity was associated with integration of the xylose-utilization genes. SSF presents a different sugar profile than the prior sugar mixture in that glucose is released slowly by cellulase enzyme throughout the fermentation. This might be advantageous because although glucose competes with xylose for transport into the cell [12], a constant background supply of glucose enhances xylose fermentation [17, 22]. CO_2 production during SSF of AM-PT-SG was monitored (Fig. 4) and samples were harvested at 96 h and analyzed for residual sugar and fermentation product concentrations (Table 6).

Table 4 Final xylose and product concentrations for xylose fermentations using industrial strains engineered to ferment xylose

Strain[a]	Xylose consumed (g/l)	Xylitol (g/l)	Glycerol (g/l)	Ethanol (g/l)	$Y_{ethanol}$ (g/g xylose)	$Y_{xylitol}$ (g/g xylose)	Carbon recovery (%)
YRH388[b]	24.0	11.9	0.97	5.6	0.23	0.50	99 ± 4
YRH390	25.9	11.4	0.61*	6.5	0.25	0.44	97 ± 1
YRH392	16.8*	7.3**	0.62	3.7**	0.22	0.44	96 ± 5
YRH394	16.1*	7.5*	0.68	3.4*	0.22	0.48	99 ± 7
YRH396	29.4*	12.9	0.90	7.8**	0.27*	0.44	98 ± 4
YRH400	30.2*	14.7*	0.52*	7.2*	0.24	0.49	97 ± 1
YRH403	17.2*	9.0	0.86	3.1*	0.18**	0.53	97 ± 4

Fermentations were performed in batch under anaerobic conditions using YP + 50 g/l xylose

Mean values were determined from three to four biological replicates and standard deviations were typically < 10% of the mean

* Value is significantly different ($p < 0.05$) from YRH388[c]

** Value is significantly different ($p < 0.01$) from YRH388[c]

[a] All strains contain the *XYL1/XYL2/XKS1* expression cassette integrated into the genome

[b] Haploid laboratory strain (CEN.PK2-1C genetic background) included for comparison

Table 5 Final sugar and product concentrations for glucose and xylose co-fermentations using industrial strains engineered to ferment xylose

Strain[a]	Xylose consumed (g/l)	Xylitol (g/l)	Glycerol (g/l)	Ethanol (g/l)	$Y_{ethanol}$ (g/g sugar)	$Y_{xylitol}$ (g/g xylose)	Carbon recovery (%)
YRH390	32.1	19.6	4.4	30.8	0.37	0.60	102 ± 2
YRH396	33.4	17.7	5.9	32.9	0.38	0.52	105 ± 8
YRH400	14.9	11.6	4.5	30.5	0.45	0.79	111 ± 7
YRH403	18.4	12.0	4.3	28.1	0.40	0.65	104 ± 3

Fermentations were performed in batch under anaerobic conditions using YP + 50 g/l glucose and 50 g/l xylose

Mean values were determined from three biological replicates and standard deviations were typically < 10% of the mean

Glucose was completely consumed

[a] All strains contain the *XYL1/XYL2/XKS1* expression cassette integrated into the genome

Each engineered strain outperformed the isogenic control (parent) strain as measured by final ethanol concentration, xylose consumption, and CO_2 production. Ethanol production was improved 13–17% for the engineered versus parent strains.

Discussion

Six industrial yeast strains were successfully engineered for xylose fermentation by integrating the necessary genes for conversion of xylose to xylulose-5-P (i.e., *XYL1*, *XYL2*, and *XKS1*). Chromosomal integration is preferred for industrial fermentations because of its associated genetic stability. The response of the engineered strains to growth on xylose varied considerably. Ethanol yields on xylose cultures were comparable with results reported by other studies using non-adapted strains (recently reviewed in [20]). However, much of the xylose was converted to the side-product xylitol, which lowered the final ethanol yields. The four superior xylose fermenting strains were further evaluated on a glucose (50 g/l) and xylose (50 g/l) mixture. All of the cultures fermented xylose in addition to glucose and the final yields were on average 20% higher than could be accounted for solely from fermentation of the glucose. Much of the consumed xylose was converted to xylitol, an observation that is consistent with previous studies [20]. Still, the selected xylose-fermenting strains produced 13–17% more ethanol from ammonia pretreated switchgrass without any decrease in fermentation rate (compared to the parent strain). Therefore, this study demonstrates that for industrial *Saccharomyces* strains engineered for xylose fermentation, genetic background is important for optimizing ethanol productivity. However, it is also likely that further strain modifications will be required to increase ethanol production by decreasing xylitol formation.

The strains in this study represent the original integration isolates and have not been further improved by mutagenesis or prolonged growth or adaption on xylose medium. Even without further modification, aerobic growth rates for these strains were considerably better than growth rates reported for other un-optimized industrial

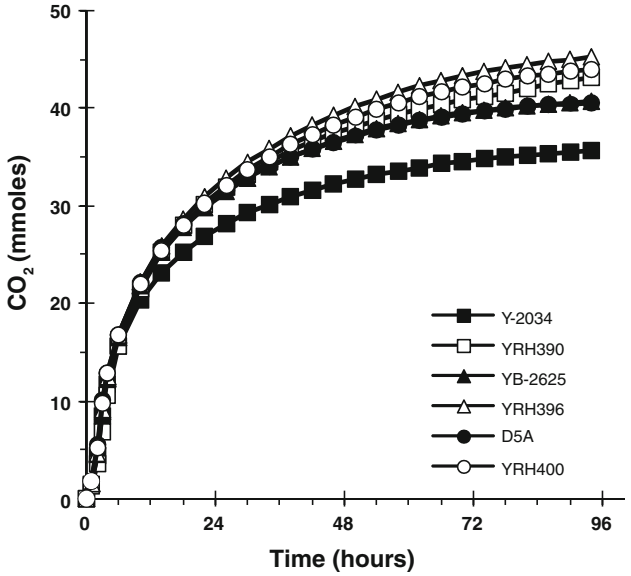

Fig. 4 Fermentation of ammonia pretreated switchgrass. Recombinant strains engineered to ferment xylose were cultured under anaerobic conditions using ammonia pretreated switchgrass hydrolysate. Data shown represent the mean CO_2 production from experiments performed in triplicate (except for strains Y-2034 and YRH400 where CO_2 production data for the third replicate was not obtained due to loss of communication with the remote sensor used to monitor pressure)

Table 6 Final sugar and product concentrations for fermentation of ammonia pretreated switchgrass for engineered and native yeast strains

Strain	Xylose[a] (g/l)	Xylitol (g/l)	Glycerol (g/l)	Ethanol (g/l)
Y-2034	9.2	8.2	0.7	13.7
YRH390[b]	7.6	7.2	1.7**	16.0*
YB-2625	9.2	8.3	1.0	14.9
YRH396[b]	7.0**	8.0	1.6**	16.8
D5A	9.1	8.5	0.8	15.0
YRH400[b]	7.8*	7.6*	1.4**	17.1*

Simultaneous saccharification and fermentation was performed in batch under anaerobic conditions using a 10% solids loading of ammonia pretreated switchgrass and ended at 96 h

Mean values were determined from three biological replicates and standard deviations were < 10% of the mean

* Value is significantly different ($p < 0.05$) from the parent strain

** Value is significantly different ($p < 0.01$) from the parent strain

[a] Xylose remaining after fermentation

[b] Strain contains the chromosomally integrated *XYL1/XYL2/XKS1* expression cassette

strains (0.01–0.05 h^{-1}) [13, 21, 33]. YRH396, the fastest growing strain, had a growth rate of 0.17 h^{-1} on SC5X medium. Strains with chromosomally integrated XR, XDH, and XK genes have been reported with growth rates in this range, but only after adaption, mutagenesis and/or additional genetic modifications [28, 33]. YRH396 was also one of the best xylose-fermenting strains in this study. However, xylose aerobic growth rates and fermentation properties were uncorrelated; a fact that needs to be considered when using aerobic cultures to select for improved xylose utilization.

In an attempt to better understand the reason for the differences between strains, we measured XR and XDH activity (data not shown). While it may seem reasonable that activity of these enzymes limits xylose metabolism, the literature is conflicted. Using isogenic strains, it has been shown that the level of XR and XDH activity does affect xylose fermentation [13]. In this study, comparing strains of differing genetic background, we did not see a correlation between the level of enzyme activity and either the rate or extent of xylose consumption. This observation suggests that factors independent of XR and XDH activity are responsible for the differences in xylose utilization seen in this study. These differences might result from varying levels of PPP activity and/or xylose transport among different genetic backgrounds and this hypothesis is under investigation.

The strains chosen for this study were also selected for their mating properties. Specifically, they are diploid and reported to form 4-spore asci. The exception to this criterion is D5A, which has been reported to be monosomic for chromosome III and display an α mating-type [3]. For the remaining strains, it should be possible to isolate a xylose-fermenting haploid with a stable mating-type, due to deletion of the *HO* endonuclease gene, which is required for mating-type switching in haploid *Saccharomyces* yeasts [30]. We expect that the ability to generate xylose-fermenting haploids of stable mating-type from these strains will be useful for incorporating additional modifications. The ability to use classical genetic techniques will also be beneficial for investigating the genetic and metabolic differences responsible for the range of growth rate and xylose fermentation observed with these strains.

In summary, six new xylose-fermenting *Saccharomyces* strains were constructed from industrial parent strains. The genetic background strongly influenced the aerobic growth rate, xylose consumption, ethanol yield, and xylitol yield when cultured on xylose or glucose/xylose mixtures. The three best strains were evaluated on ammonia pretreated switchgrass using SSF. The engineered strains readily fermented the biomass-derived sugars and produced 13–17% more ethanol than the parent strains.

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