ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Effects of Aeration on Growth, Ethanol and Polyol Accumulation by *Spathaspora passalidarum* NRRL Y-27907 and *Scheffersomyces stipitis* NRRL Y-7124

Yi-Kai Su,[1,2] Laura B. Willis,[2,3,4] Thomas W. Jeffries[2,3,4]

[1]Department of Biological Systems Engineering, University of Wisconsin, Madison, Wisconsin

[2]DOE Great Lakes Bioenergy Research Center, University of Wisconsin, Madison 53703, Wisconsin

[3]Department of Bacteriology, University of Wisconsin, Madison, Wisconsin

[4]Forest Products Laboratory, USDA Forest Service, Madison, Wisconsin;

telephone: (608) 698-4470; fax: (608) 238-1991; e-mail: twjeffri@wisc.edu

ABSTRACT: *Spathaspora passalidarum* NN245 (NRRL-Y27907) is an ascomycetous yeast that displays a higher specific fermentation rate with xylose than with glucose. Previous studies have shown that its capacity for xylose fermentation increases while cell yield decreases with decreasing aeration. Aeration optimization plays a crucial role in maximizing bioethanol production from lignocellulosic hydrolysates. Here, we compared the kinetics of *S. passalidarum* NN245 and *Scheffersomyces* (*Pichia*) *stipitis* NRRL Y-7124 fermenting 15% glucose, 15% xylose, or 12% xylose plus 3% glucose under four different aeration conditions. The maximum specific fermentation rate for *S. passalidarum* was 0.153 g ethanol/g CDW · h with a yield of 0.448 g/g from 150 g/L xylose at an oxygen transfer rate of 2.47 mmol O_2/L h. Increasing the OTR to 4.27 mmol O_2/L h. decreased the ethanol yield from 0.46 to 0.42 g/g xylose while increasing volumetric ethanol productivity from 0.52 to 0.8 g/L h. Both yeasts had lower cells yields and higher ethanol yields when growing on xylose than when growing on glucose. Acetic acid accretions of both strains correlated positively with increasing aeration. *S. passalidarum* secreted lower amounts of polyols compared to *S. stipitis* under most circumstances. In addition, the composition of polyols differed: *S. passalidarum* accumulated mostly xylitol and *R,R*-2,3-butanediol (BD) whereas *S. stipitis* accumulated mostly xylitol and ribitol when cultivated in xylose or a mixture of 12% xylose and 3% glucose. *R,R*-2,3-BD accumulation by *S. passalidarum* during xylose fermentation can be as much as four times of that by *S. stipitis*, and *R,R*-2,3-BD is also the most abundant byproduct after xylitol. The ratios of polyols accumulated by the two species under different aeration conditions and the implications of these observations for strain and process engineering are discussed.

Biotechnol. Bioeng. 2015;112: 457–469.

KEYWORDS: fermentation byproducts; fermentation kinetics; oxygen transfer rate; polyol dehydrogenase; *Spathaspora passalidarum*; xylose

Introduction

Converting lignocellulosic feedstocks into biofuels, such as ethanol, can temper the demand for gasoline and decrease CO_2 accumulation when properly integrated with cultivation of diverse native grasses (Tilman et al., 2006), sustainable agriculture (Goldemberg and Guardabassi 2009; Schmer et al., 2008), and silviculture (Dwivedi et al., 2012). Furthermore, when used as a fuel for transportation, ethanol from lignocellulosic feedstocks can reduce greenhouse gas emissions by 88% relative to gasoline, which is much greater than the 18% reduction realized from corn ethanol (Farrell et al., 2006; Schmer et al., 2008). Using cellulosic feedstocks can greatly improve greenhouse gas emissions during biofuel production on marginal lands (Gelfand et al., 2013). Hydrolysis of cellulose and hemicellulose generates a mixture of glucose, xylose, arabinose, and other monosaccharides. However, the native *Saccharomyces cerevisiae*, which has been used for centuries in alcohol production and which can readily ferment the glucan portion of lignocellulose, cannot metabolize xylose unless engineered to express functional xylose assimilation pathways (Fujii et al., 2011; Kim et al., 2013b; Kuyper et al., 2005; Ma et al., 2012; Sanchez et al., 2010). Another approach has been to use native pentose-fermenting yeasts, such as *Scheffersomyces* (*Pichia) stipitis* or *Scheffersomyces* (*Candida*) *shehatae*, either in their native forms

Correspondence to: T.W. Jeffries
Contract grant sponsor: Heart of Wisconsin (Wisconsin Rapids)
Contract grant sponsor: DOE Great Lake Bioenergy Research Center (DOE Office of Science)
Contract grant number: BER DE-FC02-07ER64494
Received 7 March 2014; Revision received 11 August 2014; Accepted 19 August 2014
Accepted manuscript online 28 August 2014;
Article first published online in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25445/abstract).
DOI 10.1002/bit.25445

or following directed evolution or metabolic engineering (Hughes et al., 2012; Jeffries and Jin 2004; Li et al., 2012).

One of major challenges for ethanol production from xylose by native pentose-fermenting yeasts is controlling the oxygen supply to maximize ethanol production. Too much oxygen leads to aerobic growth and low ethanol yield (Rizzi et al., 1989); insufficient oxygen slows the fermentation rate, increases xylitol accumulation and causes poor ethanol productivity. Under strictly anaerobic conditions, both *S. stipitis* and *S. shehatae* are only able to grow for one doubling before fermentation and cell growth stop (Du Preez 1994; Slininger et al., 1991).

Several studies have tested oxygen transfer rates (OTR) ranging from 0.7 to 8.6 mmol O_2/L h to understand the correlation between aeration and fermentation performance in native pentose fermenting yeasts (Du Preez 1994; Sreenath et al., 1986). An OTR ranging from 1.75 to 5 mmol/L h was reported as optimal for *S. stipitis* with 5% sugar (Grootjen et al., 1991; Guebel et al., 1991; Laplace et al., 1991). The optimal dissolved oxygen tension (DOT) of *S. stipitis* and *S. shehatae* less than 1% of saturation (about 0.08 mg/L = 2.5 μmol O_2/L), which is below the detection limit of dissolved oxygen sensors, but even so, the physiological responses of these strains could be different at such low DOT levels (Dellweg et al., 1989; Du Preez et al., 1989). *S. stipitis* does not produce ethanol when the DOT is above 3 μmol O_2/L (Rizzi et al., 1989) and a constant specific oxygen uptake rate (q_{OUR}) of 0.3 mmol O_2/g CDW · h can increase the ethanol yield to 0.44 g/g (Dellweg et al., 1989).

A study on *S. shehatae* showed that a q_{OUR} of 1.19 mmol O_2/g per cell weight (CDW) was able to maximize ethanol yield (0.327 g/g) with an average ethanol productivity of 2.2 g ethanol/L h in a fed-batch fermentor containing 10% xylose (Fromanger et al., 2010). Recently, an oxygen transfer coefficient (K_La) from 2.3 to 4.9 h^{-1} was suggested for *S. stipitis* cultivated on 90 g/L of xylose. In this case K_La corresponded to an OTR from 0.55 to 1.17 mmol O_2/L h at 30°C. (Silva et al., 2012). Unrean and Nguyen (2012) used an elementary mode analysis to predict that an OTR of 1.8 mmol/L h was optimal for *S. stipitis* in a 1-L fermentor containing 15 g/L of glucose and 5 g/L of xylose. Slininger et al. (2014) suggested that *S. stipitis* ethanol production is growth associated and that ethanol production can occur even when the growth rate is as high as 0.5 h^{-1}; the requisite dissolved oxygen necessary to support that rate of growth would be at least 1–3 mg O_2/L (or 31–93 μmole O_2/L) at 25°C.

Whereas *S. cerevisiae* can convert glucose to ethanol under anaerobic conditions, in most cases, engineered *S. cerevisiae* strains have a relatively high xylitol production compared to native xylose-fermenting yeasts and have been reported to require some oxygen during xylose fermentation (Jin et al., 2004). The reason engineered *S. cerevisiae* and native-xylose fermenting yeast need oxygen to ferment xylose is not clear, but it could result from an oxygen requirement for certain key biosynthetic reactions under strict anaerobic conditions (Andreasen and Stier 1953; Fornairon-Bonnefond et al., 2002; Jahnke and Klein 1983; Rosenfeld et al., 2003) or from a need to maintain cofactor balance during xylose assimilation (Jin et al., 2004). During xylose assimilation, *S. stipitis*' xylose reductase (XR) can use either NADH or NADPH as a cofactor, but it has a higher affinity for NADPH. The next enzyme in this pathway, xylitol dehydrogenase (XDH), which converts xylitol into xylulose, depends on NAD^+ almost exclusively (Liang et al., 2014). The different reactivities of the two enzymes with their respective cofactors can deplete NAD^+ and accumulate NADH. If this were the sole basis for the inhibition of growth under anaerobic conditions, then *S. stipitis* should grow anaerobically on glucose; however, this has not been demonstrated for more than one cell division. Engineering *S. cerevisiae* for xylose assimilation through heterologous expression of *S. stipitis* XR, XDH, and xylulokinase (XKS) also creates dependence on respiration in *S. cerevisiae* when xylose is the sole carbon source (Jin et al., 2004; Souto-Maior et al., 2009). Global gene expression and metabolic flux analyses on an engineered *S. cerevisiae* growing on xylose anaerobically suggested that the slower ATP formation from xylose as compared to glucose limits the anaerobic growth on xylose by *S. cerevisiae*, which is possibly due to the higher ATP requirement for xylose transporters coupled to lower ATP yields from xylose fermentation (Sonderegger et al., 2004). In contrast, using a mutated *S. stipitis* XR with increased K_m for NADPH resulted in 70% xylitol reduction from 50 g/L xylose (Jeppsson et al., 2006). In addition, engineering a mutated XR from *Candida tenuis* with a higher affinity for NADH over NADPH into *S. cerevisiae* reduced the cofactor imbalance and resulted in a 42% increase in ethanol yield (Petschacher and Nidetzky 2008).

One consequence of cofactor imbalance is seen in byproduct formation. It has been reported that small amounts of 2,3-butanediol (BD) and acetoin are as fermentation byproducts during ethanol production by *S. cerevisiae* (Neish 1950), and the genes responsible for 2,3-BD formation in *S. cerevisiae* have been characterized (Gonzalez et al., 2000; Gonzalez et al., 2010). Ligthelm et al. (1988) first reported the accumulation of pentitols (arabitol, ribitol, and xylitol) and glycerol from 40 g/L of glucose (or xylose) by native pentose-fermenting yeasts. But native pentose-fermenting yeasts can accumulate much higher levels of byproducts compared to glucose fermentation by *S. cerevisiae*. The ethanol yield from glucose by *S. cerevisiae* is about 0.48–0.49 in lab scale but the ethanol yield from xylose by native pentose-fermenting yeast ranges from 0.4 to 0.45. This lower yield has been attributed mainly to oxygen requirements for growth and higher cell yields (Slininger et al., 2014). However, no studies have investigated the accumulation of 2,3-butanediol and acetoin in native pentose-fermenting yeast.

A recently isolated native pentose-fermenting yeast, *Spathaspora passalidarum* NN245, is capable of fermenting xylose faster than glucose and can co-utilize glucose, xylose, and cellobiose as carbon sources (Hou 2012; Long et al., 2012; Nguyen et al., 2006). *S. passalidarum* and *S. stipitis* are closely related from both taxonomic and environmental perspectives. Both are found in the intestines of beetle larvae

and adults, and both belong to the "CUG" clade of yeasts that substitute serine for leucine when encountering a CUG in their messenger RNA (Wohlbach et al., 2011). Their likely evolution in association with beetles living inside of rotting wood probably accounts for their capacities to metabolize a wide range of lignocellulosic sugars and their capacities to grow at low oxygen tensions (Garcia-Ochoa and Gomez 2009; Nguyen et al., 2006). However, subsequent to the isolation of *S. passalidarum*, few publications have described its cellular physiology with respect to the oxygen requirements for ethanol production and the correlation between carbon source and polyol accumulations. In the present study, we not only investigated the fermentation performance of *S. passalidarum* and *S. stipitis* on glucose, xylose, and sugar mixtures as functions of aeration rates but also measured polyol accumulation including 2,3-BDs and pentitols during fermentation with glucose, xylose or a mixture of glucose and xylose.

Materials and Methods

Yeast Inoculum Preparation, Media

Spathaspora passalidarum NN245 (NRRL Y-27907) and *Scheffersomyces stipitis* NRRL Y-7124 were used in this study. Cultures were first streaked from -80°C stocks and transferred on either YPX or YPD agar plate containing (g/L): agar, 20; yeast extract, 10; peptone, 20; and either xylose, 20 or glucose, 20 and then cultivated for 24 h at 30°C. Cells from fresh plates were transferred into a 1-L Erlenmeyer flask containing 200 ml liquid broth composed of the following nutrients (g/L): xylose (or glucose), 40; peptone, 20; yeast extract, 10. The 1-L culture was grown at 30°C and 200 rpm overnight to reach an OD_{600} of 40, or about 7 g/L cell dry weight (CDW), prior to inoculation. Once the pre-cultures reached this concentration, cells were harvested by centrifugation at 5000 rpm for 3 min and suspended in distilled, deionized H_2O. The inoculation volume was 4% of the final total volume (the volume of cell suspension plus the volume of the medium); thus, 2 ml of cell suspension was used for 50-ml shake flask fermentation and 3 ml of cell suspension was used for 75-ml shake flask fermentation with a target initial cell concentration of about 1.5 g CDW/L ($OD_{600} \approx 15$). Modified Slininger (MS) medium is based on Slininger's optimal medium (Slininger et al., 2006) except that MS medium contained 3.25 g/L of urea, and no purine, pyrimidine, or thioctic acid were used.

Effects of Aeration on Fermentation

To determine the maximum ethanol production from xylose by *S. passalidarum*, various aeration rates were applied to evaluate its fermentative ability at 25°C. Tests of aeration rates were performed in 125-ml polycarbonate Erlenmeyer flasks (GeneMate) fitted with 0.22 μm pore membrane filters for oxygen transfer. Flasks containing 50 or 75 ml of MS medium were shaken at 110 or 150 rpm to achieve four different aeration conditions (Table I). Aeration conditions 2 and 3 have been used to evaluate or screen the performance of pentose-fermenting yeasts (Slininger et al., 2006; Sreenath et al., 1986). The carbon sources consisted of 150 g/L (15%) glucose, 150 g/L (15%) xylose, or a mixture of 120 g/L (12%) xylose and 30 g/L (3%) glucose. Duplicate flasks in each aeration condition were used and a control experiment was performed at the same time with *S. stipitis* in each aeration condition. Xylose fermentations used inocula grown on xylose as the carbon source while glucose and mixed sugar fermentations used inocula grown on glucose. Samples (0.8 ml) were withdrawn daily from each flask for HPLC and cell density determinations. Fermentation rates and yields were based on calculations over the first 48-h to avoid effects of product inhibition or substrate limitation that could interfere with the kinetic values. The sulfite-oxidation method (Garcia-Ochoa and Gomez 2009) was used to determine the oxygen transfer rate (OTR) in each fermentation condition.

Table I. Aeration conditions used in these experiments.

Aeration condition	Liquid volume in a 125-ml flask (ml)	Agitation (rpm)	OTR (mmol O_2/L h)
1	75	110	2.47
2	75	150	3.65
3	50	110	4.27
4	50	150	4.73

Culture Sampling and Analyses of Sugar, Ethanol and Major Fermentation Byproducts

Cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) with an Agilent 8453 spectrophotometer. Yeast CDW was estimated by correlating OD_{600} (5–100 OD_{600}) with gravimetric measurements of dried cells from the same cultures: about 2–10 ml of cell suspension was filtered through 0.2 μm hydrophilic polyethersulfonate membrane (PALL, New York) and the cell mass was dried as described by Postma et al. (1989). Under the conditions used, 1 OD_{600} equals 0.1101 (±0.01) g/L of CDW for *S. passalidarum* NRRL Y-27907and 0.133 (±0.007) g/L of CDW for *S. stipitis* NRRL Y-7124. All sugars and fermentation metabolites including D-arabitol, xylitol, ribitol, and glycerol were determined by high performance liquid chromatography (HPLC) using a Hitachi auto sampler, pump and refractive index detector, and a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at 65°C (Fromanger et al., 2010). The mobile phase was 0.005 M H_2SO_4 at flow rate of 0.6 mL/min. External standardization was used with frequent calibration of response factors.

Analyses of Acetoin and 2,3-Butanediol During Fermentation

S. cerevisiae normally produces 2,3-butanediol (BD) consisting of ~67% of *R,R*-2,3-BD, 33% *meso*-2,3-BD and less than 1% *S,S*-2,3-BD (Gonzalez et al., 2000; Romano et al., 2003).

We assume less than 1% of *S,S*-2,3BD in the total 2,3-BD production for the native pentose-fermenting and the major composition in 2,3-BD will be *R,R*-2,3-BD and *meso*-2,3-BD. Acetoin, *meso*-2,3-BD, and *R,R*-2,3-BD were confirmed by HPLC-MS and the quantitative analyses of these products, along with polyols, were determined by HPLC (Jiang et al., 2012) using the same methods mentioned in the previous section.

Results

Fermentation Performance With Various OTRs in Modified Slininger (MS) Medium

To determine the potential ethanol productivity of *S. passalidarum* NN245 at various aeration rates, fermentations were carried out at 25°C using 15% glucose, 15% xylose, or a sugar mixture of 12% xylose and 3% glucose under four different aeration levels (Fig. 1). For glucose fermentations, cell growth rate, sugar utilization, ethanol productivity decreased after 48 h and ethanol accumulation on glucose exceeded accumulation on xylose only at the lowest aeration level. On the other hand, for xylose and mixed sugar fermentations, the cell growth rate and ethanol productivity did not slow down until 72 h. On glucose, CDW rose quickly and then dropped after 48 h. With xylose or mixed sugar fermentations, cell yield was much lower from the outset, and cell concentration stayed steady throughout the fermentation. Peak ethanol concentrations occurred between 72 and 120 h, depending on aeration conditions. The highest ethanol concentration was 58.3 g/L from mixed sugar fermentation (12% xylose, 3% glucose) at the lowest aeration level (2.47 mmol O_2/L h). Similarly, the lowest aeration also produced the highest ethanol yield, 0.45 g/g from 150 g/L xylose This was also the condition in which *S. passalidarum* exhibited the highest specific ethanol productivity, 0.153 g ethanol/g CDW · h (Table SI).

To better understand our findings with *S. passalidarum* at various aeration conditions, we conducted a comparable experiment under the same conditions with *S. stipitis* NRRL-Y7124 (Fig. S1). The peak ethanol concentration attained with *S. stipitis* was 53.3 ± 0.3 g/L at 2.47 mmol O_2/L h from 15% xylose. On glucose, we observed a similar rapid but incomplete sugar consumption accompanied with rapid initial cell growth followed by a decline in CDW after 48 h. Under most circumstances, *S. passalidarum* produced more ethanol compared to *S. stipitis*, except when glucose was the sole carbon source at an OTR of 3.65 or 4.73 mmol O_2/L h. The relatively lower ethanol yield on xylose was mirrored by a relatively higher cell yield in comparison to that observed with *S. passalidarum*. The fermentation rates and yields of the two yeasts were similar, but overall *S. passalidarum* performed better than *S. stipitis* under the lower aeration conditions, especially when xylose was the sole carbon source.

We compared the fermentation kinetics of *S. passalidarum* NN245 and *S. stipitis* NRRL Y-7124 at 48 h as functions of the oxygen transfer rates (Fig. 2). The specific growth rates and cell yields of *S. passalidarum* on glucose were clearly higher than observed with *S. stipitis*, especially for the cell yield at OTR of 4.73 mmol O_2/L h, and with both yeasts, these values increased as aeration increased (Fig. 2A and B). However, when the mixed sugar was used, *S. stipitis* showed higher specific growth rates compared to those of *S. passalidarum*. During xylose or mixed sugar fermentations, ethanol yields of both strains dropped by 14–16% as the aeration rate increased from 2.47 to 4.73 mmol O_2/L h. *S. passalidarum's* specific ethanol productivity (q_E) was roughly 50% higher on xylose than on glucose over the entire range of aeration rates examined. By comparison, its volumetric rate of ethanol production from xylose increased with aeration while the volumetric rate with glucose was essentially constant. On the other hand, for *S. stipitis*, its q_E responded to the increasing aeration with an optimized OTR at 3.65 mmol O_2/L h with xylose as the carbon source. For q_E on glucose, xylose or a mixture of the two sugars, *S. stipitis* exhibited a range between 0.114 and 0.137 g ethanol/g CDW · h while *S. passalidarum* showed a wider range of 0.089–0.153 g ethanol/g CDW · h (Table SI). The ethanol productivities of *S. stipitis* exhibited similar trends compared to those from *S. passalidarum*, but the rates from xylose and mixed sugar were about 15–20% lower than those from *S. passalidarum* (Fig. 2).

Polyol and Acetic Acid Accumulation Under Different Aeration Rates

With *S. passalidarum*, the accumulation of xylitol tripled from about 1 g/L to about 3 to 4 g/L when low amounts of glucose were present. Similar but less pronounced trends were observed with *S. stipitis* (Fig. 2). We found no clear correlation between aeration and arabitol or ribitol accumulation (Fig. 3). In contrast, acetic acid increased when aeration increased with both of the yeast species and each of the three sugar substrates (Fig. S2). More acetic acid accumulated on glucose as compared to xylose or mixed sugar fermentations. When the carbon source was xylose or a mixture of 12% xylose and 3% glucose, *S. passalidarum* produced only 40–50% as much acetic acid as from glucose alone. The highest acetic acid production was 2.11 g/L from 15% glucose at 4.73 mmol O_2/L h. *S. passalidarum* produced much less ribitol compared to *S. stipitis* during xylose or mixed sugar fermentation (Figs. 3 and S2). *S. stipitis* converted roughly 3.3% of the available xylose into ribitol and accumulated as much as 5 g/L. In contrast, *S. passalidarum* generally produced less than 0.6 g/L of ribitol and always less than 1 g/L under all of the conditions tested. *S. passalidarum* accumulated more arabitol than ribitol, and the arabitol concentration could be twice as much as that of ribitol when glucose was the sole carbon source (Fig. 3A, D, G, and J). But when mixed sugars were used, *S. passalidarum* produced arabitol and ribitol in similar quantities. By comparison, *S. stipitis* tended to accumulate much more ribitol than arabitol during xylose or mixed sugar fermentation and the final ribitol concentration could be 5 to 6 times higher than the final arabitol concentration when pure xylose was the sole carbon source (Fig. S2 B, F, H, and K). When

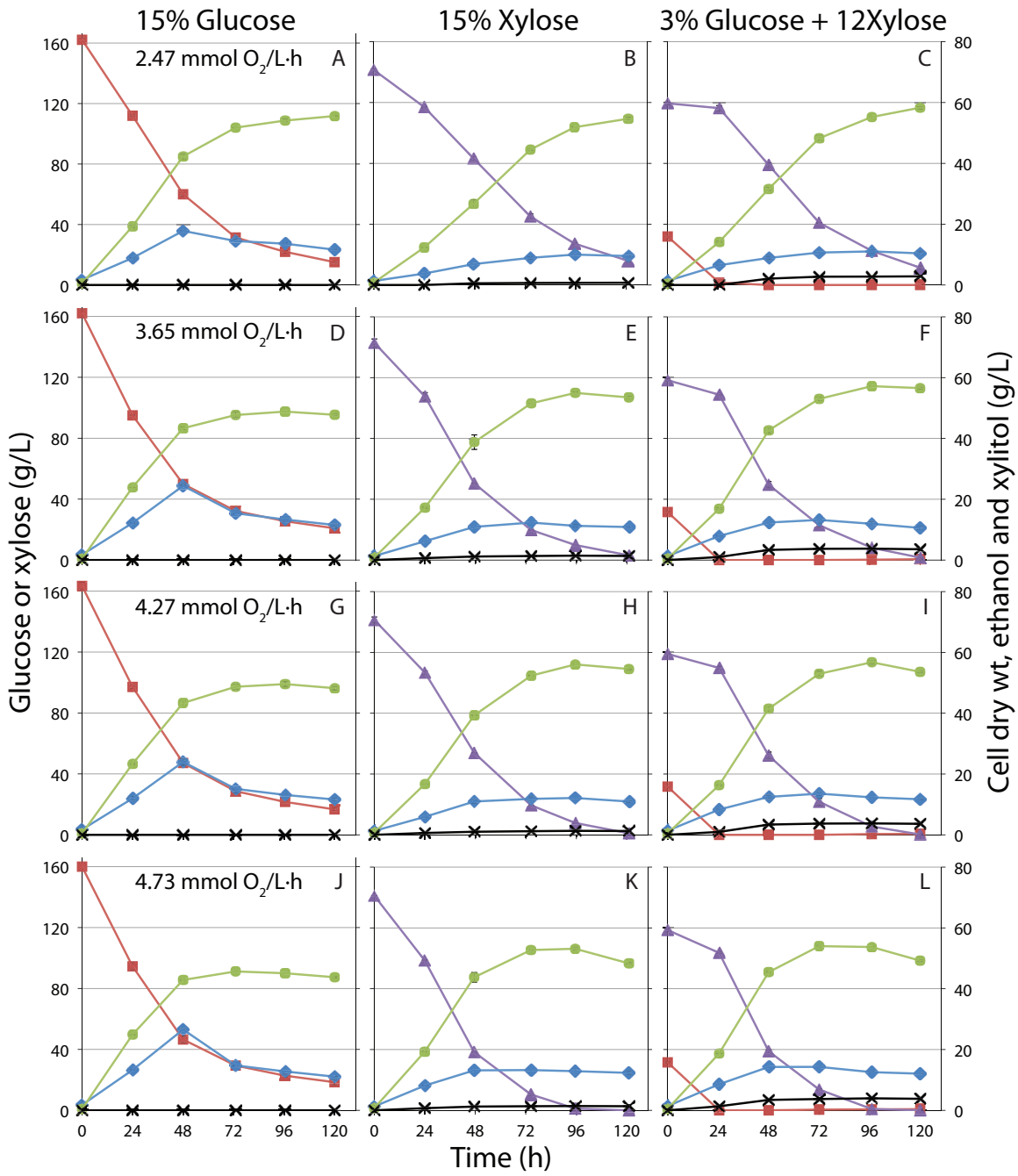

Figure 1. Ethanol production from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *Spathaspora passalidarum* NN245 in duplicate shake flasks under different aerations (2.47, 3.65, 4.27, and 4.73 mmol O_2/L h). Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol.

glucose was the sole carbon source, *S. stipitis* accumulated similar amounts of arabitol and ribitol at an OTR of 2.47, and higher arabitol accumulation was observed when OTR was higher than 3.65 mmol O_2/L h.

S. passalidarum and *S. stipitis* both produced glycerol, acetoin, *meso*-2,3-butanediol (BD), and *R,R*-2,3-BD during fermentation (Figs. 4 and S3). With *S. passalidarum*, all of these byproducts accumulated to higher concentrations as aeration increased. Notably, *S. passalidarum* produced four times more *R,R*-2,3-BD than *S. stipitis*, which was the highest byproduct besides xylitol during the xylose and the mixed sugar fermentations. A spike of acetoin production from 0.45 to 0.91 g/L occurred in the first 24 h during glucose and the mixed sugar fermentations but not when xylose was the sole carbon source. *S. passalidarum* accumulated more glycerol when the carbon source was glucose or mixed sugars than

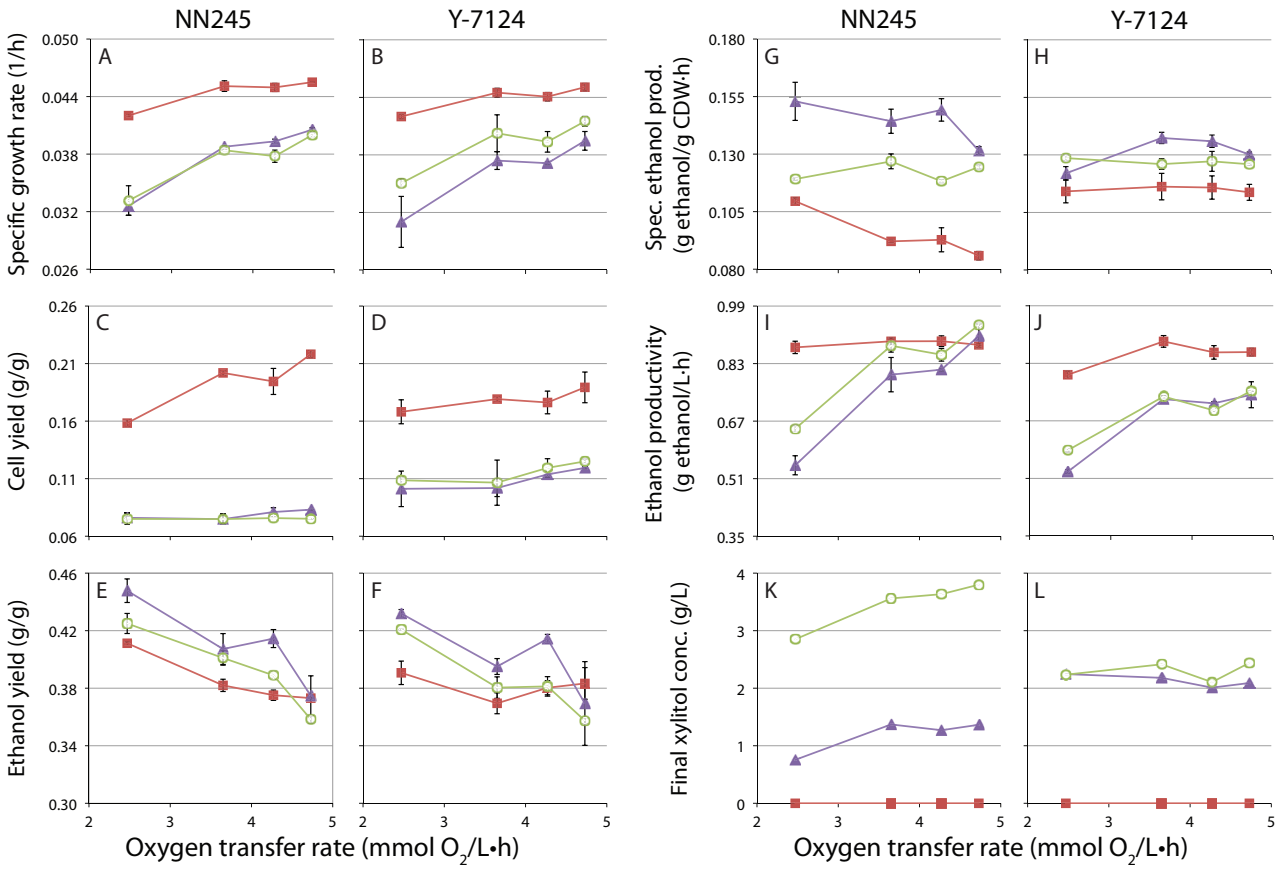

Figure 2. Fermentation kinetics at first 48 h and final xylitol concentration from 15% glucose (■), 15% xylose (▲), and a mixture of 12% xylose and 3% glucose (○) by *S. passalidarum* NN245 and *S. stipitis* NRRL-Y7124 under various aerations in duplicate shake flasks. Error bars depict the range of values.

when cultivated on xylose. Moreover, during the first 24-h fermentation, a faster accumulation of glycerol was found in the first 24 h from mixed sugar fermentation than from glucose fermentation. For *S. stipitis* (Fig. S3), during glucose fermentation, the accumulations of glycerol and acetoin correlated with increasing aeration. The accumulation of polyols by *S. passalidarum* appeared to be different than that of *S. stipitis* (Fig. S4). The ratio of pentitol to total polyol yield (RPPY, mM/mM) can vary from 0.11 to 0.25 for *S. passalidarum*, but the value was around 0.5 for *S. stipitis* when glucose was the carbon source. More striking, the RPPY of *S. passalidarum* ranged 0.28–0.42, but the RPPY of *S. stipitis* was about 0.8 when xylose was the sole carbon source. A summary of acetic acid and polyol accumulation by *S. passalidarum* and *S. stipitis* is shown in Table II.

Discussion

Fermentation Kinetics and Oxygen Transfer Rates

In the present experiments, the inocula were cultured with yeast extract and peptone (YP), which was the same method described by (Long et al., 2012) because (i) YP medium has been widely use for inoculum preparation; and (ii) only about 4–6 h is needed for *S. stipitis* to adjust the expression level of genes related to glycolytic pathway and PPP during fermentation after transition of cultures based on previous transcription microarray study (Jeffries and Van Vleet 2009). If the inocula had been cultivated in the same MS medium as used for the fermentation trials, it would have reduced the transition, but likely would not have altered the reported rates or yields.

For both yeasts, aeration exhibited a greater effect on cell yields than on specific growth rates during glucose fermentation, but during xylose or mixed sugar fermentation, aeration exerted more influence on specific growth rates than on cell yields (Fig. 2A–D). Also, both yeasts had lower cells yields when growing on xylose than when growing on glucose and higher ethanol yields on xylose than on glucose except at the highest aeration rate examined (Fig. 2E and F). The most likely explanation for the observations among cell yields, specific growth rates, ethanol yield in response to various aerations is that net ATP yields are substantially lower when either yeast is growing on xylose than when growing on glucose, and that the ATP yield from xylose is lower with *S. passalidarum* than with *S. stipitis*. Consequently, cells need to

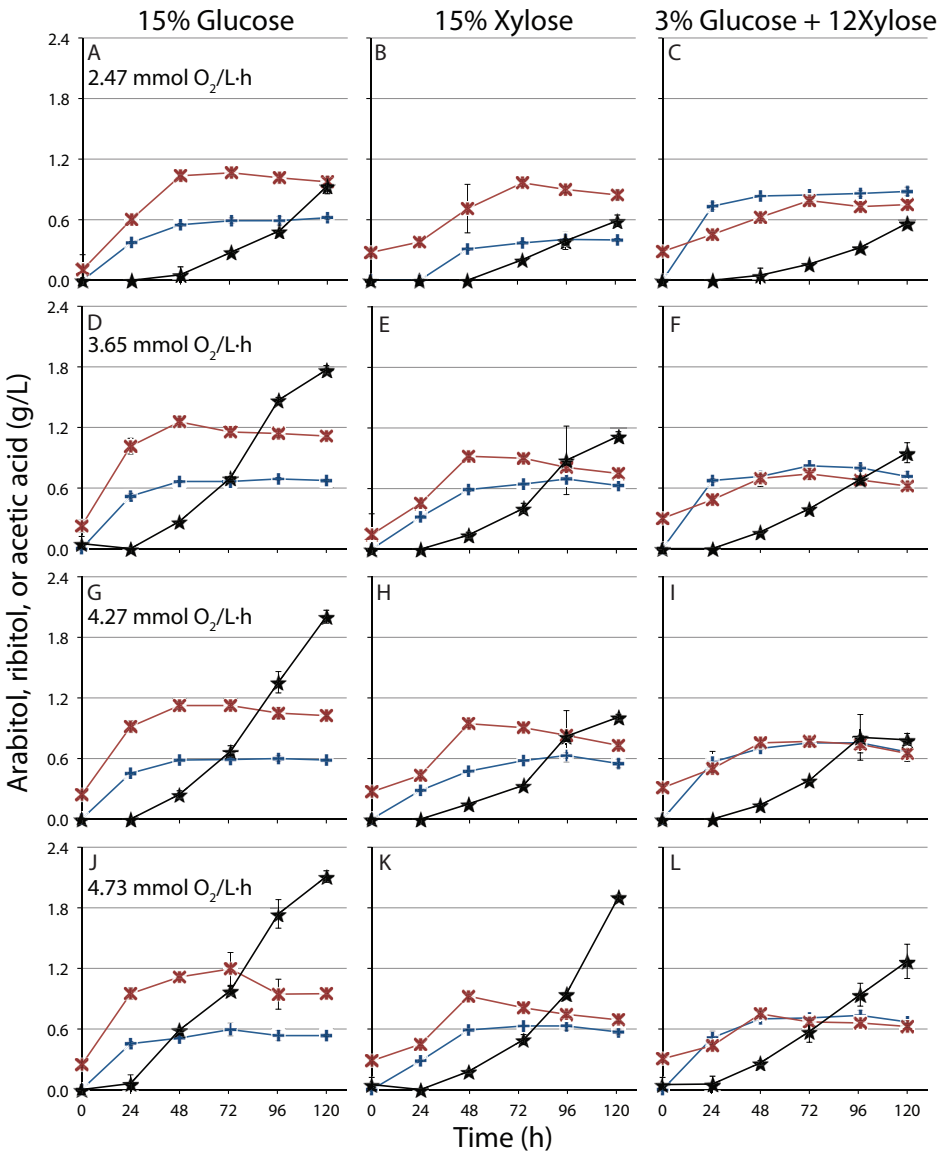

Figure 3. The formation of arabitol, ribitol and acetic acid from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *S. passalidarum* NN245 under different aerations (2.47, 3.65, 4.27, and 4.73 mmol O_2/L h) in duplicate shake flasks. Symbols: ✖, arabitol; ✚, ribitol; ★, acetic acid.

consume more carbon to make the same amount of cell mass. Two metabolic steps could account for the lower ATP yield: sugar uptake by symporters and the xylulose phosphorylation step (Fig. 5). The energy expended per mole of sugar should be similar regardless of whether a C5 or C6 sugar is taken up via symport; the same would hold true for phosphorylation, but the C moles of carbon would be 17% less in each step.

For both strains and for all three carbon sources, our results are consistent with previous studies in *S. stipitis* and *S. shehatae*, which also showed reduced ethanol yield with increasing aeration (Du Preez et al., 1989; Silva et al., 2012). The q_E we observed was similar to what had been reported with *S. stipitis* (Dellweg et al., 1989; Laplace et al., 1991) Interestingly, *S. passalidarum* showed a 1.5 to 2-fold increase in the q_E from xylose compared to glucose due to a 52–63% decrease in cell yield. A similar result was also observed by Long *et al.* (2012), who reported a q_E on xylose 3.4-fold higher than on glucose in a 2-L fermenter trial. When xylose or mixed sugar was the carbon source, both yeast improved q_E when aeration increased, which was also reported previously for *S. stipitis* (Du Preez et al., 1989; Silva et al., 2012).

By comparing the overall fermentation kinetics (Fig. 2 and Table SI), the data suggested that the two yeasts respond differently to the carbon sources and aeration rates. Based on our results, when xylose was the sole carbon source, the ethanol yield of *S. passalidarum* ranged from 0.46 to 0.42 with an OTR corresponding to 2.47 and 4.27 mmol O_2/L h. These

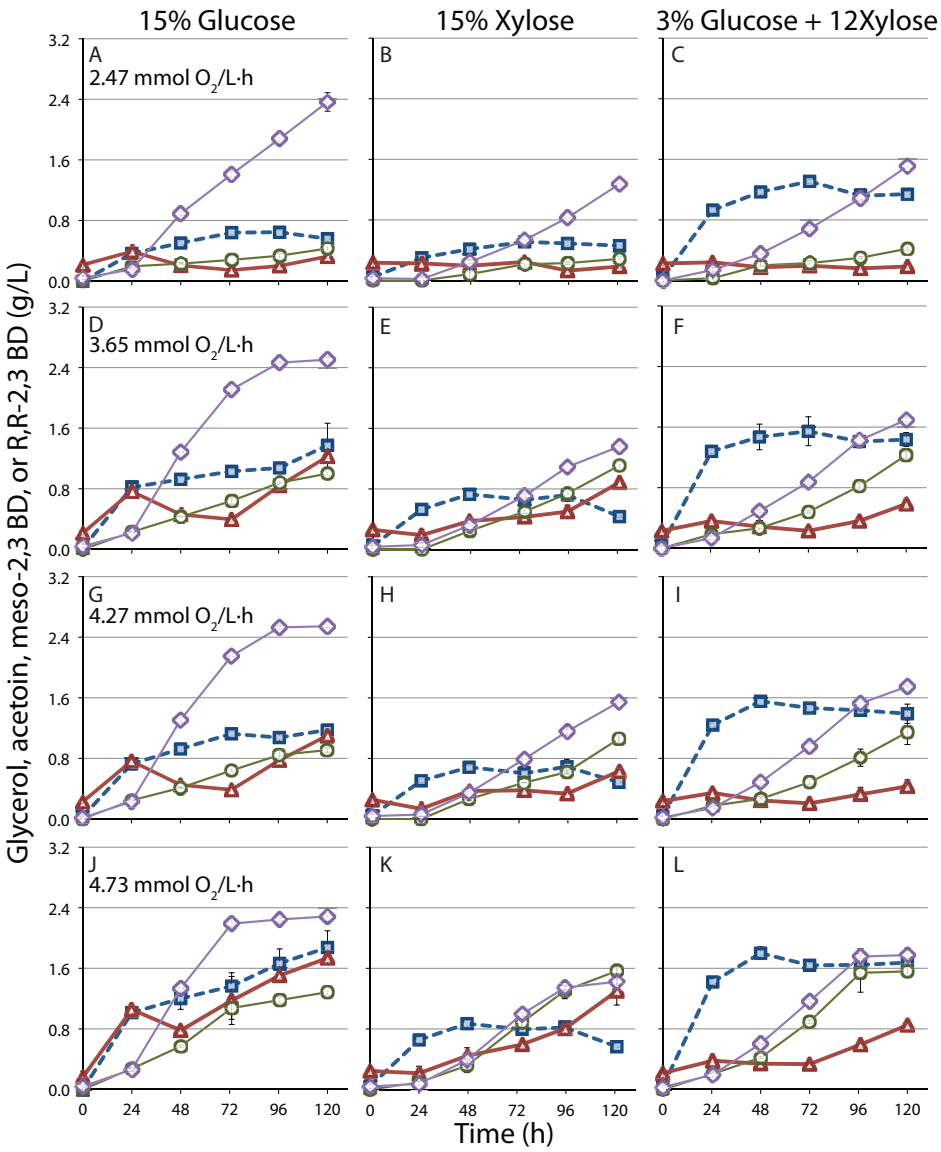

Figure 4. The accumulation of glycerol, acetoin, *meso*-2,3 butanediol (*meso*-2,3BD) and *R,R*-2,3 Butanediol (*R,R*-2,3 BD) from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *S. passalidarum* NN245 under different aerations (2.47, 3.65, 4.27, and 4.73 mmol O_2/L h) in duplicate shake flasks. Symbols: ■, glycerol; △, acetoin; ○, *meso*-2,3-BD; ◇, *R,R*-2,3-BD.

yields compare favorably to ethanol yields from xylose that are obtained with metabolically engineered *Saccharomyces cerevisiae* (Bengtsson et al., 2009; Madhavan et al., 2009; Shen et al., 2012), but fermentations of pure xylose solutions are not generally reported for engineered *S. cerevisiae*, and even more rarely are they reported for sugar concentrations as high as those used here. Increasing aeration dramatically increased ethanol productivity from 0.52 to 0.8 g/L h. Over this range specific ethanol productivity (q_E) was maintained at ≈ 0.15 g/g CDW · h. If we divide OTR values by the cell mass and assume (i) no change in OTR during fermentation; and (ii) oxygen diffusing into the medium was completely consumed by yeasts (DOT = 0), a q_{OUR} at 48 h can be calculated around 0.33–0.39 mmol O_2/g CDW · h for *S. passalidarum*. With the same method, a q_{OUR} ranging from 0.34 to 0.4 mmol O_2/g CDW · h can be calculated for *S. stiptis*, which is very close to the value obtained with *S. passalidarum*. Our *S. stipitis's* q_{OUR} values match some of the studies that were previously reported in the literature, which was about 0.3 mmol O_2/g CDW · h for the xylose fermentation (Dellweg et al., 1989; Grootjen et al., 1991; Guebel et al., 1991; Laplace et al., 1991). Our numbers are higher than some reported from previous research, possibly because of differences in the sugar concentration (cf. Unrean and Nguyen 2012), or medium composition and fermentation configurations (cf. Silva et al.,

Table II. Summary of acetic acid and polyols acccumulations[a], g/L.

Carbon source	Xylitol	Ribitol	Arabitol	Glycerol	Acetoin	*R,R*-2,3 BD	*meso*-2,3 BD	Ratio of 2,3BD[b]	Acetic acid
Spathaspora passalidarum NN245									
15% glucose	nd[c]	0.5–0.6	~ 1	0.6–1.9	0.3–1.7	2.3–2.5	0.4–1.3	1.8–5.6	1–2
15% xylose	0.8–1.4	0.4–0.6	0.7–0.9	0.4–0.6	0.2–1.3	1.3–1.6	0.3–1.6	0.9–4.5	0.6–1.9
12% xylose, 3% glucose	2.9–3.8	0.7–0.9	0.6–0.8	1.1–1.7	0.2–0.9	1.5–1.8	0.4–1.6	1.4–3.6	0.6–1.2
Scheffersomyces stipitis NRRL Y-7124									
15% glucose	nd	~2.7	2.8–3.5	0.8–1.5	0.7–2	0.5–0.7	1.3–1.5	0.4–0.5	1.8–4.7
15% xylose	2–2.3	3.6–5.4	0.6–1	~0.2	0.2–0.6	0.2–0.4	0.4–1	0.3–0.4	0.4–1.1
12% xylose, 3% glucose	2.2–2.4	2.4–4.2	0.7–1	0.3–0.6	~0.2	~0.2	0.5–0.6	0.3–0.4	0.5–0.9

[a]OTR values ranged from 2.47 to 4.73 mmol O_2/L h after 120-h fermentation from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% gluocse in duplicate shake flasks by *Spathaspora passalidarum* NN245 and *Scheffersomyces stipitis* NRRL Y-7124.
[b]The ratio of *R,R*-2,3 BD to *meso*-2,3 BD.
[c]Not detectable.

2012). A summary of the oxygen requirement for xylose fermentation is presented in Table III.

Polyol and Acetic Acid Accumulation

The native yeasts studied here can ferment both glucose and xylose through concerted glycolytic and pentose phosphate pathways. In the process, they can form arabitol and ribitol in addition to xylitol (Fig. 5) We observed increases in xylitol accumulation by *S. passalidarum* on mixed sugars with a low amount of glucose compared to the results on pure xylose, however cell yields and specific growth rates were very similar under both conditions (Fig. 2C and K), suggesting that the increase in xylitol production was not triggered by insufficient DOT. We postulated that the small amount of glucose might have down-regulated glycolytic genes and enzymes in *S. passalidarum*. The difference between final xylitol concentrations produced by *S. passalidarum* and *S. stipitis* during mixed sugar fermentation (Fig. 2K and L) suggested small amounts of glucose had different effects on the two strains. Polyol accumulation during yeast fermentation usually serves as an alternative way to regenerate cofactors such as $NAD(P)^+$ when $NAD(P)H + H^+$ is in excess. Acetic acid accumulation can occur through the partial oxidation of acetaldehyde when $NAD(P)H + H^+$ is limited. Polyol production started early in the fermentation and once formed, they were not readily re-assimilated under low aeration conditions. By comparison, acetic acid accumulation increased with increasing aeration and cell growth. The

Table III. Fermentation parameters for ethanol production from xylose.

Yeast	Type[a]	Xylose (g/L)	Ethanol (g/L)	$Y_{X/S}$ (g/g)	$Y_{E/S}$ (g/g)	Q_E (g/L · h)	q_E	DOT (%)	OTR	q_{OUR}	Reference
S. passalidarum											
NN245	F	140	27–40	~0.08	~0.43	~0.72	~0.15	ns	2.5–4.7	0.33–0.39	This study[b]
S. shehatae											
CBS 2779	FB	129	44	0.07	0.34	0.99	0.34	0.7	ns	ns	du Preez et al. (1989)
ATCC 22984	FB	100	48.1	~0.13	~0.33	~2.2	~0.22	ns	ns	1.19	Fromanger et al. (2010)
ATCC 22984	Fr	50	~19.5	0.09	0.39	ns	0.15	~0	3.9	ns	Laplace et al. (1991)
ATCC 22984	Fr	90	26	0.05	0.28	1.06	*0.12*	ns	8.5	ns	Sreenath et al. (1986)
S. stipitis											
NRRL Y-7124	Fr	ns	ns	~0.11	0.39	ns	0.12	ns	3.75	0.3	Dellweg et al. (1989)
NRRL Y-7124	Fr	15b	ns	ns	~0.4	ns	0.24	0	0.7	~0.3	Delgenes et al. (1989)
NRRL Y-7124	F	50	ns	0.34	0.16	0.5	0.41	ns	5	ns	Guebel et al. (1991)
NRRL Y-7124	Fr	50	~21.5	0.12	0.43	ns	0.12	0	1.75	ns	Laplace et al. (1991)
NRRL Y-7124	Fr	50	~18	0.39	ns	ns	0.12	<1	3.75	ns	Rizzi et al. (1989)
NRRL Y-7124	Fr	90[c]	~27	~0.14	~0.29	~0.3	ns	ns	0.6–1.8	ns	Silva et al. (2012)
NRRL Y-7124	F	140	~35	~0.11	~0.39	~0.73	~0.13	ns	2.5–4.7	0.34–0.4	This study[b]
CBS 7126	FB	129	~28	~0.15	~0.35–0.4	1.6–1.8	0.3–0.4	0.2–0.7	Ns	ns	du Preez et al. (1989)
CBS 6054	C	50	ns	ns	0.48	ns	0.2	0	<1	ns	Skoog and Hahnhagerdal (1990)
BCC15191	Fr	ns	ns	ns	0.4	0.25	ns	ns	1.8[d]	ns	Unrean and Nguyen (2012)

$Y_{X/S}$, cell yield; $Y_{E/S}$, ethanol yield; Q_E, volumetric ethanol productivity; q_E, specific ethanol productivity, g/g DCW · h; DOT, dissolved oxygen tension; OTR, oxygen transfer rate, mmol O_2/L · h; q_{OUR}, specific oxygen uptake rate, mmol O_2/g DCW · h; ns, not stated.
[a]F, flask; FB, fed-batch; Fr, fermentor, C, continuous culture.
[b]All the value were calculated at first 48 h.
[c]contained 15 g/L of glucose.
[d]This result was based on elementary model analysis.

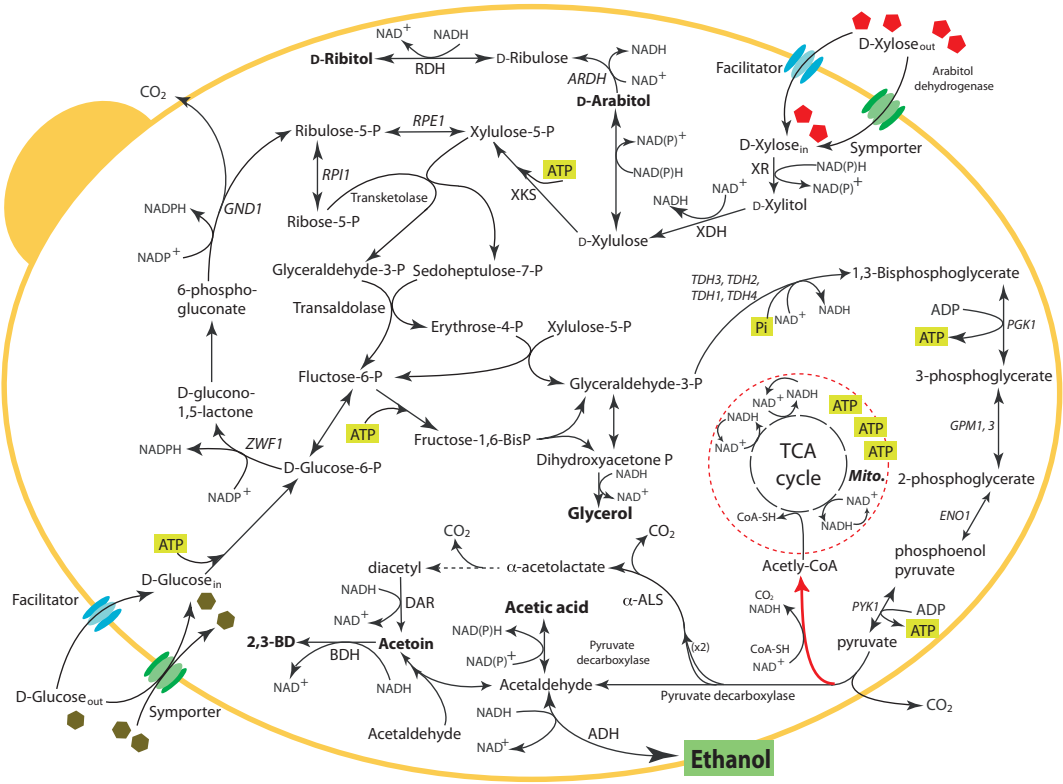

Figure 5. The proposed metabolic pathway including glycolysis, pentose phosphate pathway, polyol accumulation, and a simplified tricarbolic cycle in a native pentose-fermenting yesat. α-ALS, α-acetolactate synthase; ADH, alcohol dehydrogenase; BDH, butanediol dehydrogenase; DAR, diacetyl reductase; XR, xylose reductase; XDH, xylitol dehydrogenase; XKS, xylulose kinase; *ARDH* (arabitol-2-dehydrogenase); *ENO1*, enolase (2-phosphoglycerate dehydratase); *GND1*, 6-phosphogluconate dehydrogenase; *GPM*, phosphoglycerate mutase; *PGK1*, phosphoglycerate kinaes; *PYK1*, pyruvate kinase; *RPE1*, D-ribulose-5-phosphate 3-epimerase; *RPI1*, ribose-5-phophate isomerase; *TDH*, glceraldehyde-3-phosphate dehydrogenase; *ZWF1*, glucose-6-phosphate dehyrogenase; Mito., mitochodria; the arrow with black dashline indicates a spontaneous reaction.

highest accumulations by both yeasts were from glucose at an OTR of 4.73 mmol O_2/L h. Increasing aeration increased cell yield and increased the demand for NADPH, which could be met by making more acetic acid.

Interestingly, *S. passalidarum* accumulated more D-arabitol than ribitol during xylose or glucose fermentation; but *S. stipitis* accumulated much more ribitol than D-arabitol during xylose or mixed sugar (12% xylose, 3% glucose) fermentation. One possible explanation could be a difference in how the two yeasts handle the cofactor imbalance created in the conversion of xylose to xylulose. The xylose reductase activity of *S. passalidarum* shows a higher affinity for NADH than seen with *S. stipitis* (Hou, 2012). This could result in a higher flux for xylose assimilation under low aeration conditions. A less efficient glycolytic flux in *S. stipitis* NRRL Y-7124 could lead to greater depletion in NAD^+ in the cytosol (due to lower recycling of NAD^+ by reduction of acetaldehyde to ethanol). This would result in greater conversion of arabitol to ribitol by an oxidoreducatase to regenerate NAD^+. During glucose fermentation, *S. passalidarum* accumulated about 80% of polyols derived from pyruvate while *S. stipitis* only generated about 40% of non-pentitol polyols (Fig. 5). If the hypothesis of more ATP being generated with glucose compared to xylose is correct, this observation suggests a more active glycolysis with xylose in *S. passalidarum* than in *S. stipitis*. We grew *S. passsalidarum* NN245 in minimal defined medium [medium composition based on (Verduyn et al., 1992)] in an anaerobic chemostat fitted with a copper oxygen scavenger, and observed only xylitol and ribitol (no arabitol) accumulation as major byproducts during fermentation of 5% xylose (data not shown). This implies that under strict anaerobic conditions, *S. passalidarum* was also forced to further convert arabitol to ribitol.

S. cerevisiae exhibited a ratio of *R,R*-2,3 BD to *meso*-2,3 BD around 2 (Gonzalez et al., 2000) while *S. passalidarum* exhibited a wide range of ratios (0.9–5.6) depending on substrates and aeration while the ratio of *R*,R-2,3 BD to *meso*-2,3 BD from *S. stipitis* was only about 0.3–0.5. These differences in major byproduct formation during fermentation (Table II) between *S. passalidarum* and *S. stipitis* could also be attributed to the more efficient glycolysis pathway of *S. passalidarum* than that of *S. stipitis*. We examined the literature and the genomes of *S. passalidarum* and *S. stipitis* to identify genes that might encode enzymes that could carry out the

conversion of aldo-ketones to polyols. NAD(P)$^+$-dependent cytoplasmic polyol dehydrogenases catalyzing reversible reactions between polyols and aldo-ketones have been intensively investigated in acetic acid bacteria (Cheng et al., 2005) but not in fungi. The first D-arabitol dehydrogenase characterized in fungi is Ard1 (Accession no. P43066), a D-arabitol-2-dehydrogenase (ribulose-forming) from *Candida albicans*, which produces large amounts of D-arabitol from glucose (Wong et al., 1993). A short-chain D-arabitol dehydrogenase in *S. stipitis* (*SsARDH*) with 84% identity to Ard1 of *C. albicans* was reported (Hallborn et al., 1995). Related research indicates these genes reversibly convert D-arabitol to D-ribulose (Murray et al., 1995). A homologue to *Ss*ARDH in *S. passalidarum* (Accession no. EGW30261) with 85.6% similarity can be found through UniProt (http://www.uniprot.org). The ARDH found in *S. passalidarum* could also responsible for the conversion between D-arabitol to D-ribulose. However, the gene or protein responsible for conversion between D-xylulose to D-arabitol (D-arabitol-4-dehydrogenase) has not yet been identified. A ^{13}C nuclear magnetic resonance studies in *C. albicans* suggested the *Ard1* protein may only be responsible for D-arabitol uptake because while an *ard* null mutant of *C. albicans* is unable to utilize D-arabitol, it can still synthesize D-arabitol from glucose via the same pathway compared to its wild-type parent (Wong et al., 1995).

Recently, a versatile NADP$^+$-dependent D-arabitol dehydrogenase (Ard1p) capable of reversibly catalyzing the reaction from arabitol to either ribulose or xylulose and mannitol into fructose was isolated from a plant pathogenic fungus *Uromyces fabae* (Link et al., 2005). A BLAST analysis of Ard1p on the database finds sorbitol dehydrogenases (SOR) in *S. passalidarum* (24.8% identity) and *S. stipitis* (23.8%) instead of the two short-chain arabitol dehydrogenases from both yeasts. In addition, the two sorbitol dehydrogenases from both *S. passalidarum* and *S. stipitis* along with Ard1p from *U. fabae* belong to the Zn^{2+} alcohol dehydrogenase family with NAD(P)$^+$ binding site. Phylogenic analyses also suggest that Ard1p is more closely related to the *S. passalidarum* and *S. stipitis* sorbitol dehydrogenases than to the D-arabitol-2-dehydrogenases in *S. passalidarum* and *S. stipitis* (Fig. S5). These finding suggest the possibility of an oxidoreductase in yeast using NAD$^+$ as a cofactor and converting D-xylulose into D-arabitol, which was also stated in other literature (Cheng et al., 2009; Wong et al., 1995). Two butanediol dehydrogenases from *S. cerevisiae* (Bdh1 and Bdh2) have already been confirmed and characterized (Gonzalez et al., 2000) and possible candidate butanediol dehydrogenase in *S. passalidarum* and *S. stipitis* can also be found by BLAST of their genomes database with *S. cerevisiae* in sequence database (NCBI access number EGW32915 and 34210 for *S. passalidarum*, and XP_001385027 and XP_001386484 for *S. stipitis*). These provide targets for further investigation of polyol accumulation by pentose fermenting yeasts, and may help to elucidate the key differences in hexose and pentose metabolism that lead to differential byproduct formation in *S. passalidarum* and *S. stipitis* under a range of aeration conditions. A recent publication by Kim et al. (In press) successfully introduced a xylose assimilation pathway (*XYL1*, *XYL2*, and *XYL3*) from *S. stipitis* into a pyruvate decarboxylase deficient *S. cerevisiae* to increase the flux into 2,3-BD accumulation. The mutant *S. cerevisiae* produced *R,R*-2,3-BD dominantly (>97%). For *S. passalidarum*, *R,R*-2,3-BD, the second highest byproduct during xylose fermentation and the highest byproduct during glucose fermentation at lower aeration (2.47–3.65 mmol O_2/L h) suggests *S. passalidarum* could be a promising native pentose-fermenting yeast for metabolic engineering for 2,3-butanediol fermentation.

Conclusion

Our findings, together with data from our previous studies, confirm the preferential xylose fermentation by *S. passalidarum*, and more importantly, the specific fermentation rate is a least 1.5-fold higher on xylose than on glucose in flask-scale. A q_{OUR} of 0.33–0.39 mmol O_2/g CDW · h for *S. passalidarum* was shown when OTR ranged from 2.5 to 4.7 mmol O_2/L h with 150 g/L of xylose. Based on the fermentation kinetic data presented above our findings suggest that, under the oxygen transfer rates applied in this study, *S. passalidarum* could be a better native xylose-fermenting yeast than *S. stipitis*. *S. passalidarum* produced a smaller proportion of pentitols (20–50%) in the total polyols accumulated and higher 2,3-BDs compared to *S. stipitis*, suggesting a higher glycolytic flux in *S. passalidarum*.

This work was funded in part by a grant from the Heart of Wisconsin (Wisconsin Rapids) to the University of Wisconsin-Madison and by the DOE Great Lake Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494). We thank Alan Higbee and Mick McGee for performing some of the HPLC-MS analyses; Devon Moloney and Ellyn Lepinski for sampling and data compilation; Meredith Blackwell, Louisiana State University, for providing the original culture of *Spathaspora passalidarum*; and Sarah Mahan for providing the original draft of the metabolic map.

Nomenclature

BD	Butanediol
CDW	Cell dry weight, g/L
OTR	Oxygen transfer rate, mmol O_2/L h
PPP	Pentose phosphate pathway
Q_E	Ethanol productivity, g ethanol/L
q_E	Specific ethanol productivity, g ethanol/g CDW · h
q_{OUR}	Specific oxygen utilization rate, mmol O_2/g CDW · h
RPPY	Ratio of pentitol yield to polyol yield
$Y_{E/S}$	Ethanol yield, g/g
$Y_{X/S}$	Cell yield, g/g

References

Andreasen AA, Stier TJB. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J Cell Comp Physiol 41(1):23–36.

Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund MF. 2009. Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 2:9.

Cheng H, Li Z, Jiang N, Deng Z. 2009. Cloning, purification and characterization of an NAD-dependent d-arabitol dehydrogenase from acetic acid bacterium, *Acetobacter suboxydans*. Protein J 28(6): 263–272.

Cheng HR, Jiang N, Shen A, Feng YJ. 2005. Molecular cloning and functional expression of D-arabitol dehydrogenase gene from *Gluconobacter oxydans* in *Escherichia coli*. FEMS Microbiol Lett 252(1):35–42.

Delgenes JP, Moletta R, Navarro JM. 1989. Fermentation of D-xylose, D-glucose, L-arabinose mixture by *Pichia stipitis* - Effect of the oxygen transfter rate on fermentation performance. Biotechnology and Bioengineering 34(3): 398–402.

Dellweg H, Rizzi M, Klein C. 1989. Contolled limited aeration and metabolic regulation during the prduction of ethanol from D-xylose by *Pichia stipitis*. J Biotechnol 12(2):111–122.

Du Preez JC. 1994. Process parameters and environmental-factors affecting D-xylose fermentation by yeasts. Enzyme Microb Technol 16(11):944–956.

Du Preez JC, Vandriessel B, Prior BA. 1989. D-Xylose fermentation by *Candida shehatae* and *Pichia stipitis* at low dissolved oxygen level in fed-batch cultures. Biotechnol Lett 11(2):131–136.

Dwivedi P, Bailis R, Alavalapati J, Nesbit T. 2012. Global warming impact of E85 fuel derived from forest biomass: A case study from southern USA. Bioenergy Res 5(2):470–480.

Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM. 2006. Ethanol can contribute to energy and environmental goals. Science 311(5760):506–508.

Fornairon-Bonnefond C, Demaretz V, Rosenfeld E, Salmon JM. 2002. Oxygen addition and sterol synthesis in *Saccharomyces cerevisiae* during enological fermentation. J Biosci and Bioeng 93(2):176–182.

Fromanger R, Guillouet SE, Uribelarrea JL, Molina-Jouve C, Cameleyre X. 2010. Effect of controlled oxygen limitation on Candida shehatae physiology for ethanol production from xylose and glucose. J Ind Microbiol Biotechnol 37(5):437–445.

Fujii T, Yu G, Matsushika A, Kurita A, Yano S, Murakami K, Sawayama S. 2011. Ethanol production from xylo-oligosaccharides by xylose-fermenting *Saccharomyces cerevisiae* expressing beta-xylosidase. Biosci Biotechnol Biochem 75(6):1140–1146.

Garcia-Ochoa F, Gomez E. 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. Biotechnol Adv 27(2):153–176.

Gelfand I, Sahajpal R, Zhang XS, Izaurralde RC, Gross KL, Robertson GP. 2013. Sustainable bioenergy production from marginal lands in the US Midwest. Nature 493(7433):514–517.

Goldemberg J, Guardabassi P. 2009. Are biofuels a feasible option? Energy Policy 37(1):10–14.

Gonzalez E, Fernandez MR, Larroy C, Sola L, Pericas MA, Pares X, Biosca JA. 2000. Characterization of a (2R,3R)-2,3-butanediol dehydrogenase as the *Saccharomyces cerevisiae* YAL060W gene product—Disruption and induction of the gene. J Biol Chem 275(46):35876–35885.

Gonzalez E, Rosario Fernandez M, Marco D, Calam E, Sumoy L, Pares X, Dequin S, Biosca JA. 2010. Role of *Saccharomyces cerevisiae* oxidoreductases Bdh1p and Ara1p in the metabolism of acetoin and 2,3-butanediol. Appl Environ Microb 76(3):670–679.

Grootjen DRJ, Vanderlans R, Luyben K. 1991. Effects of the aeration rate on the fermentation of glucose and xylose by *Pichia stipitis* CBS 5773. Enzyme Microb Technol 12(1):20–23.

Guebel DV, Cordenons A, Nudel BC, Giulietti AM. 1991. Influence of oxygen transfer rate and media composition on fermentation of D-xylose by *Pichia stipitis*. J Ind Microbiol 7(4):287–291.

Hallborn J, Walfridsson M, Penttila M, Keranen S, Hahnhagerdal B. 1995. A short-chain dehydrogenase gene from *Pichia stipitis* having D-arabinitol dehydrogenase activity. Yeast 11(9):839–847.

Hou X. 2012. Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94(1):205–214.

Hughes SR, Gibbons WR, Bang SS, Pinkelman R, Bischoff KM, Slininger PJ, Qureshi N, Kurtzman CP, Liu SQ, Saha BC. and others. 2012. Random UV-C mutagenesis of *Scheffersomyces* (formerly *Pichia*) *stipitis* NRRL Y-7124 to improve anaerobic growth on lignocellulosic sugars. J Ind Microbiol Biotechnol 39(1):163–173.

Jahnke L, Klein HP. 1983. Oxygen requirement for formation and activity of the squalene epoxidase in *Saccharomycse cerevisiae*. J Bacteriol 155(2):488–492.

Jeffries TW, Jin YS. 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63(5):495–509.

Jeffries TW, Van Vleet JRH. 2009. *Pichia stipitis* genomics, transcriptomics, and gene clusters. FEMS Yeast Res 9(6):793–807.

Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal R, Gorwa-Grauslund MF. 2006. The expression of a *Pichia stipitis* xylose reductase mutant with higher K(M) for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 93(4):665–673.

Jiang LQ, Fang Z, Guo F, Yang LB. 2012. Production of 2,3-butanediol from acid hydrolysates of Jatropha hulls with *Klebsiella oxytoca*. Bioresour Technol 107:405–410.

Jin YS, Laplaza JM, Jeffries TW. 2004. *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. Appl Environ Microbiol 70(11):6816–6825.

Kim SJ, Seo SO, Jin YS, Seo JH. 2013a. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. Bioresource Technology 146:274–281.

Kim SR, Lee KS, Kong II, Lesmana A, Lee WH, Seo JH, Kweon DH, Jin YS. 2013b. Construction of an efficient xylose-fermenting diploid *Saccharomyces cerevisiae* strain through mating of two engineered haploid strains capable of xylose assimilation. J Biotechnol 164(1):105–111.

Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Research 5(10):925–934.

Laplace JM, Delgenes JP, Moletta R, Navarro JM. 1991. Alcoholic fermentation of glucose and xylose by *Pichia stiptis*, *Candida shehatae*, *Saccharomyces cerevisiae* and *zymomonas mobilis*—oxygen requirement as a key factor. Appl Microbiol Biotechnol 36(2):158–162.

Li Y, Park J-y, Shiroma R, Ike M, Tokuyasu K. 2012. Improved ethanol and reduced xylitol production from glucose and xylose mixtures by the mutant strain of *Candida shehatae* ATCC 22984. Appl Biochem Biotechnol 166(7):1781–1790.

Liang M, Damiani A, He QP, Wang J. 2014. Elucidating xylose metabolism of *Scheffersomyces stipitis* for lignocellulosic ethanol production. ACS Sustainable Chem Eng 2(1):38–48.

Ligthelm ME, Prior BA, Dupreez JC. 1988. The oxygen requirements of yeasts for the fermentation of D-xylose and D-glucose to ethanol Appl Microbiol Biotechnol 28(1):63–68.

Link T, Lohaus G, Heiser I, Mendgen K, Hahn M, Voegele RT. 2005. Characterization of a novel NADP(+)-dependent D-arabitol dehydrogenase from the plant pathogen *Uromyces fabae*. Biochem J 389:289–295.

Long TM, Su YK, Headman J, Higbee A, Willis LB, Jeffries TW. 2012. Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast spathaspora passalidarum. Appl Environ Microbiol 78(16):5492–5500.

Ma M, Liu ZL, Moon J. 2012. Genetic engineering of inhibitor-tolerant *Saccharomyces cerevisiae* for improved xylose utilization in ethanol production. Bioenergy Res 5(2):459–469.

Madhavan A, Tamalampudi S, Srivastava A, Fukuda H, Bisaria VS, Kondo A. 2009. Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization. Appl Microbiol Biotechnol 82(6):1037–1047.

Murray JS, Wong ML, Miyada CG, Switchenko AC, Goodman TC, Wong B. 1995. Isolation, characterization and experssion of the gene that encodes D-arabinitol dehydrogenase in *Candida tropicalis*. Gene 155(1):123–128.

Neish AC. 1950. Note on the stereoisomers of 2,3-butanediol produced by yeast. Can J Res 28b(10):660–661.

Nguyen NH, Suh SO, Marshall CJ, Blackwell M. 2006. Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum gen. sp nov* and *Candida jeffriesii sp nov*. Mycol Res 110:1232–1241.

Petschacher B, Nidetzky B. 2008. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 7:9.

Postma E, Verduyn C, Scheffers WA, Vandijken JP. 1989. Enzymic analysis of the crabtree effect in glucose-limited chmostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55(2):468–477.

Rizzi M, Klein C, Schulze C, Buithanh NA, Dellweg H. 1989. Xylose fermentation by yeasts. 5. Use of ATP balances for modeling oxygen-limited growth and fermentation of yeast *Pichia stipitis* with xylose as carbon sources. Biotechnol Bioeng 34(4):509–514.

Romano P, Granchi L, Caruso M, Borra G, Palla G, Fiore C, Ganucci D, Caligiani A, Brandolini V. 2003. The species-specific ratios of 2,3-butanediol and acetoin isomers as a tool to evaluate wine yeast performance. Int J Food Microbiol 86(1–2):163–168.

Rosenfeld E, Beauvoit B, Blondin B, Salmon JM. 2003. Oxygen consumption by anaerobic *Saccharomyces cerevisiae* under enological conditions: Effect on fermentation kinetics. Appl Environ Microbiol 69(1):113–121.

Sanchez RG, Karhumaa K, Fonseca C, Nogue VS, Almeida JRM, Larsson CU, Bengtsson O, Bettiga M, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2010. Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. Biotechnol Biofuels 3:11.

Schmer MR, Vogel KP, Mitchell RB, Perrin RK. 2008. Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci U S A 105(2):464–469.

Shen Y, Chen X, Peng BY, Chen LY, Hou J, Bao XM. 2012. An efficient xylose-fermenting recombinant *Saccharomyces cerevisiae* strain obtained through adaptive evolution and its global transcription profile. Appl Microbiol Biotechnol 96(4):1079–1091.

Silva JPA, Mussatto SI, Roberto IC, Teixeira JA. 2012. Fermentation medium and oxygen transfer conditions that maximize the xylose conversion to ethanol by *Pichia stipitis*. Renewable Energy 37(1):259–265.

Slininger PJ, Branstrator LE, Bothast RJ, Okos MR, Ladisch MR. 1991. Growth, death, and oxygen-uptake kinetics of *Pichia stipitis* on xylose. Biotechnol Bioeng 37(10):973–980.

Slininger PJ, Dien BS, Gorsich SW, Liu ZL. 2006. Nitrogen source and mineral optimization enhance D-xylose conversion to ethanol by the yeast *Pichia stipitis* NRRL Y-7124. Appl Microbiol Biotechnol 72(6):1285–1296.

Slininger PJ, Dien BS, Lomont JM, Bothast RJ, Ladisch MR, Okos MR. 2014. Evaluation of a kinetic model for computer simulation of growth and fermentation by *Scheffersomyces (Pichia) stipitis* fed D-xylose. Biotechnol Bioeng 111(8):1532–1540.

Skoog K, Hahnhagerdal B. 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Applied and Environmental Microbiology 56(11):3389–3394.

Sonderegger M, Jeppsson M, Hahn-Hagerdal B, Sauer U. 2004. Molecular basis for anaerobic growth of *Saccharomyces cerevisiae* on xylose, investigated by global gene expression and metabolic flux analysis. Appl Environ Microbiol 70(4):2307–2317.

Souto-Maior AM, Runquist D, Hahn-Hägerdal B. 2009. Crabtree-negative characteristics of recombinant xylose-utilizing *Saccharomyces cerevisiae*. J Biotechnol 143(2):119–123.

Sreenath HK, Chapman TW, Jeffries TW. 1986. Ethanol production from D-xylose in batch fermentations with *Candida shehatae*: Process variables. Appl Microbiol Biotechnol 24(4):294–299.

Tilman D, Hill J, Lehman C. 2006. Carbon-negative biofuels from low-input high-diversity grassland biomass. Science 314(5805):1598–1600.

Unrean P, Nguyen NHA. 2012. Metabolic pathway analysis of *Scheffersomyces (Pichia) stipitis*: effect of oxygen availability on ethanol synthesis and flux distributions. Applied Microbiology and Biotechnology 94(5):1387–1398.

Verduyn C, Postma E, Scheffers WA, Vandijken JP. 1992. Effect of benzoic acid on metabolic fluxes in yeasts—a continuous culture study in the regulation of respiration and alcohol fermentation. Yeast 8(7):501–517.

Wohlbach DJ, Kuo A, Sato TK, Potts KM, Salamov AA, Labutti KM, Sun H, Clum A, Pangilinan JL, Lindquist EA. and others. 2011. Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. Proc Natl Acad Sci U S A 108(32):13212–13217.

Wong B, Leeson S, Grindle S, Magee B, Brooks E, Magee PT. 1995. D-Arabitol metabolism in *Candida albicans*—construction and analysis of mutatns lacking D-arabitol dehydrogenase. J Bacteriol 177(11):2971–2976.

Wong B, Murray JS, Castellanos M, Croen KD. 1993. D-Arabitol metabolism in *Candida albicans*—studies of the biosynthetic pathway and the gene that encodes NAD-dependent D-arabitol dehydrogenase. J Bacteriol 175(19):6314–6320.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.