

Xylose Isomerase Improves Growth and Ethanol Production Rates from Biomass Sugars for Both *Saccharomyces Pastorianus* and *Saccharomyces Cerevisiae*

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The demand for biofuel ethanol made from clean, renewable nonfood sources is growing. Cellulosic biomass, such as switch grass (*Panicum virgatum* L.), is an alternative feedstock for ethanol production; however, cellulosic feedstock hydrolysates contain high levels of xylose, which needs to be converted to ethanol to meet economic feasibility. In this study, the effects of xylose isomerase on cell growth and ethanol production from biomass sugars representative of switch grass were investigated using low cell density cultures. The lager yeast species *Saccharomyces pastorianus* was grown with immobilized xylose isomerase in the fermentation step to determine the impact of the glucose and xylose concentrations on the ethanol production rates. Ethanol production rates were improved due to xylose isomerase; however, the positive effect was not due solely to the conversion of xylose to xylulose. Xylose isomerase also has glucose isomerase activity, so to better understand the impact of the xylose isomerase on *S. pastorianus*, growth and ethanol production were examined in cultures provided fructose as the sole carbon. It was observed that growth and ethanol production rates were higher for the fructose cultures with xylose isomerase even in the absence of xylose. To determine whether the positive effects of xylose isomerase extended to other yeast species, a side-by-side comparison of *S. pastorianus* and *Saccharomyces cerevisiae* was conducted. These comparisons demonstrated that the xylose isomerase increased ethanol productivity for both the yeast species by increasing the glucose consumption rate. These results suggest that xylose isomerase can contribute to improved ethanol productivity, even without significant xylose conversion. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 669–680, 2012

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Introduction

The production of ethanol from nonfood-based biomass has the potential to alleviate the growing demand for clean fuels that do not contribute to the buildup of greenhouse gases in the atmosphere. Due to the high cellulose content and rapid growth of switch grass (*Panicum virgatum* L.), the US Department of Energy has identified it as a model herbaceous renewable energy crop. For switch grass biomass to be fermented to ethanol, it first needs to be treated to release the sugars in a two-step process termed pretreatment and hy-

drolysis. Pretreated hydrolyzed switch grass contains approximately 60% glucose and 40% xylose.¹ The ability of the organisms to completely ferment these mixed sugars to ethanol is an important aspect of economic feasibility.²

Saccharomyces cerevisiae is the most commonly used species for biofuel production; however, there are several other *Saccharomyces* species with robust growth rates and high ethanol tolerance. *Saccharomyces pastorianus* is currently used in the lager brewing industry and is responsible for 90% of the beer market.³ *S. pastorianus* is a hybrid of the traditional brewing yeasts *Saccharomyces bayanus* and *S. cerevisiae*.⁴ *S. cerevisiae* and *S. bayanus* often share environmental niches and are found together in wine and beer fermentations.^{5,6} Generally, *S. cerevisiae* grows and produces

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ethanol between 30 and 35°C,⁷ while *S. bayanus* can grow and produce ethanol at temperatures ranging from 1 to 30°C.^{8–10} The *S. pastorianus* strain has retained the ability to ferment sugars at low temperatures (8–15°C) from *S. bayanus*.^{3,11} Additionally, *S. pastorianus* can tolerate the high gravity conditions of brewing that include high ethanol and sugar concentrations^{12,13} and ferment sugars at temperatures up to 34°C.⁷ *S. pastorianus* has separate glucose and fructose transporters,¹⁴ whereas the single *S. cerevisiae* hexose transporter significantly prefers glucose to fructose.¹⁵ *S. pastorianus* has sufficient favorable growth- and ethanol-tolerance characteristics to be considered a fermentative candidate species for cellulosic ethanol production, and may provide increase tolerance to biomass sugar hydrolysate inhibitors compared with *S. cerevisiae*.

For an ethanol process to be economical, it is considered essential that the xylose from cellulosic biomass be converted to ethanol.^{2,16} As all yeasts of the genus *Saccharomyces* lack the gene that produces the enzyme (xylose isomerase),¹⁷ conversion of xylose to xylulose is necessary for carbon uptake.^{18,19} Recombinant approaches have been used to genetically modify *S. cerevisiae* to be able to metabolize xylose directly^{20,21}; however, these xylose metabolism modifications in recombinant yeasts have not demonstrated higher ethanol productivity or the high ethanol tolerances of the native *Saccharomyces*.^{16,22–24} Recently, combining xylose metabolism modifications with recombinant cellobiose use in *S. cerevisiae* resulted in improved xylose to ethanol conversion rates, matching xylose to ethanol conversions reported for *Pichia stipitis*²⁵; however, ethanol tolerance was not fully examined.²⁶ Although recombinant approaches may eventually achieve acceptable rates and conversions, the ability to sell the yeast-rich distiller solids^{27,28} will remain problematic unless the US Food and Drug Administration grants “Generally Regarded As Safe” status to the recombinant yeast strains (21 CFR Parts 170).

Alternatively, exogenous xylose isomerase has been used to convert xylose to xylulose, allowing *S. cerevisiae* to metabolize xylulose into ethanol during fermentation.^{18,19,29} These approaches have included isomerization followed by fermentation and simultaneous isomerization and fermentation.^{18,19,29,30} Yuan et al.²⁹ found that xylose was most efficiently used when it was preisomerized to xylulose before the simultaneous isomerization and fermentation step. It was unclear whether suboptimal growth conditions (40°C, pH 6) or the xylose-to-xylulose conversion was limiting ethanol production. Both the groups used immobilized xylose isomerase with high initial cell concentrations (5–250 g dry cell weight/L), where no significant cell growth in the fermentation step was reported. Additionally, these studies neglected to include in their ethanol yield calculations the sugar cost to generate the inoculum biomass. As it typically takes 2 g of sugar to produce a gram of yeast biomass,³¹ the sugar needed to generate the inoculum biomass can be significant. Also, studies have shown that growing yeast produce ethanol at rates approximately 33 times higher than resting cells,³¹ and thus improved ethanol production from xylulose maybe achieved if actively growing cells are used.

To capitalize on the high ethanol production capability of *S. pastorianus*, a simultaneous isomerization and fermentation process was investigated for actively growing low cell density *S. pastorianus* cultures, such that the conversion of sugars to ethanol was reported, accounting for biomass production. The objective of this study was to quantify the con-

version of glucose and xylose to ethanol by *S. pastorianus* under optimal growth conditions. Immobilized xylose isomerase was added to the fermentations as supplied by the manufacturer without additional modifications. Xylose isomerase is also a glucose isomerase, so to better understand the impact of the xylose isomerase on *S. pastorianus*, growth and ethanol production rates were examined in cultures provided fructose as the sole carbon with and without xylose isomerase. The effects of the immobilized enzyme activity were also investigated. Finally, side-by-side comparisons of ethanol production rates using *S. pastorianus* and *S. cerevisiae* on mixed sugars were conducted with and without xylose isomerase.

Materials and Methods

Microorganism and growth medium

S. pastorianus was obtained from Winemaker (Beaverbank, NS, Canada) and is commercially sold as Liquor Quik Super Yeast X-Press. The manufacturer classified the species as brewing yeast, most likely *S. bayanus*, which has the potential to reach 180 g/L ethanol. In our hands, the good growth characteristics of the organism at both 4 and 34°C suggested that the species was not consistent with *S. bayanus* or *S. cerevisiae*.^{7,10,32} Thus, genetic profiling was used to further characterize the species, which is described below. *S. cerevisiae* was obtained from the Lesaffre Yeast Corporation (Milwaukee, WI), sold commercially as RED STAR Active Dry Yeast.

Yeast cultures were grown on minimal medium modified from Korz et al.³³ and Sharma et al.³⁴ supplemented with 1% rich medium and 0.1 mg/mL ampicillin (Sigma). Ampicillin was included to decrease the potential for bacterial contamination,³² and its use is consistent with the starch-based and sucrose-based ethanol industrials, which allows the distiller solids to be used in cattle feed.³¹ The minimal medium contained a phosphate buffer (KH₂PO₄ and (NH₄)₂HPO₄), citric acid, magnesium sulfate, trace metals (MnCl₂·4 H₂O, Zn (CH₃COO)₂·2 H₂O, H₃BO₃, Na₂MoO₄·2 H₂O, CoCl₂·6 H₂O, CuCl₂·2 H₂O, and EDTA), and iron (III) citrate at pH 5.8. The rich medium was adapted from the Yeast/Peptone/Dextrose (YPD) medium of Gong et al.¹⁹ and contained 10 g/L yeast extract and 20 g/L peptone with 0 or 50 g/L glucose as indicated in the results section. Various concentrations of glucose, xylose, and xylose isomerase were added to the growth medium as indicated in the Results section. KH₂PO₄, (NH₄)₂HPO₄, MnCl₂·4 H₂O, Zn (CH₃COO)₂·2 H₂O, H₃BO₃, fructose, glucose, and citric acid were purchased from Fisher Scientific. Magnesium sulfate, CoCl₂·6 H₂O, iron (III) citrate, and peptone were purchased from Sigma. Yeast extract, xylose, Na₂MoO₄·2 H₂O, EDTA, and CuCl₂·2 H₂O were purchased from Acros. All chemicals were ACS certified or ≥99% pure, except the yeast extract and peptone, which were microbiological certified.

Xylose isomerase

Xylose isomerase was used in this study to reversibly isomerize xylose to xylulose. The enzyme xylose isomerase was purchased from Sigma and is marketed as glucose isomerase obtained from *Streptomyces murinus*. The original manufacturer is Novozyme Corporation under the trade name Sweetzyme IT Extra. The immobilized enzyme and supporting material was used at a concentration of 5 g/L. The immobilized

xylose isomerase enzyme is reusable at least five times under the growth conditions investigated (data not shown). Additionally, the manufacturer states that the enzyme is active for over 200 days under the glucose isomerization conditions used in the high-fructose corn syrup industry.

Mixed sugar media with xylose isomerase

Fermentation media were prepared with various sugar additions to the minimal media with the 1% rich media containing 50 g/L glucose. The glucose concentrations investigated were 0.5, 80, and 160 g/L; the xylose concentrations investigated were 0, 40, and 80 g/L; and the xylose isomerase additions were 0 and 5 g/L. Triplicate cultures were investigated for all conditions, and all combinations were examined.

Fructose and glucose media with xylose isomerase

Fermentation media were prepared with fructose or glucose additions to the minimal media with the 1% rich media containing no glucose (also no xylose). The fructose and glucose concentrations investigated separately were 80 and 160 g/L; and the xylose isomerase additions were 0 and 5 g/L. Duplicate cultures for the 80 g/L sugar conditions and triplicate cultures for the 160 g/L sugar conditions were conducted.

Side-by-side species comparison mixed sugar media with xylose isomerase

Fermentation media were prepared with various sugar additions to the minimal media with the 1% rich media containing no glucose. The glucose concentration was 160 g/L; the xylose concentrations investigated were 40 and 80 g/L; and the xylose isomerase additions were 0 and 5 g/L. Triplicate cultures were investigated for all conditions.

Inactivation of xylose activity

The xylose isomerase activity was inactivated by autoclaving the immobilized enzyme dry for 15 min at 121°C in a standard laboratory autoclave on the liquid cycle. Other methods of inactivation were also examined. Other methods that were successful included autoclaving the enzyme in water for 15 min at 121°C in a standard laboratory autoclave on the liquid cycle, microwaving the dry enzyme for 1 min on high (100% power in a 1000 Watt microwave), and microwaving enzyme in water for 1 min on high (100% power in a 1000 Watt microwave). Methods that failed to inactivate the enzyme included incubating the enzyme with 6 M guanidine HCl for 1 h and incubating the enzyme in 100% ethanol for 1 h. Enzyme inactivation was confirmed by contacting the enzyme with the minimal media containing glucose and xylose for 10 h (no cells). If no xylulose was detected in the solution, the inactivation was considered successful. Positive and negative control samples of the active enzyme and no enzyme were run in parallel. For the positive control, the xylulose concentration reached over 5 g/L in 10 h. For the negative control, no xylulose was detected after 10 h of incubation.

Fermentations

Overnight cultures of yeast grown aerobically were used to inoculate the growth media containing the prepared sugar levels; thus the starting sugar concentrations were approxi-

mately 10% lower than the medium due to the dilution of the medium sugars by the inoculum. Fermentations were performed at 30°C in 250 mL shake flasks with a 100-mL working volume. Cultures were grown in an orbital shaker and agitated at 130 rpm. Culture flasks were sealed with rubber stoppers that contained a one-way air valve and a sampling syringe. The one-way air valve allowed carbon dioxide to escape from the shake flasks, while minimizing the introduction of oxygen, thus providing an anaerobic environment for ethanol production. The inoculating cell densities were approximately 0.05 OD, which corresponds to a dry cell weight (dcw) of 0.025 g dcw/L for both the species.

Analytical methods

Cell growth was measured by absorbance at 600 nm with a Spectronic 20 Genesys (Spectronic Instruments, UK). Sugars and ethanol concentrations were determined throughout the fermentations. Samples (1.5 mL) were taken periodically. These samples were centrifuged to separate the yeast and the supernatant was collected. The supernatant was filtered through a 0.2- μ m syringe filter. Calibration standards were also filtered.

For the mixed sugar and inactive enzyme fermentations, samples and standards were analyzed by high-pressure liquid chromatography (HPLC). Glucose, xylose, xylulose, and ethanol concentrations were measured with a 1200 series Agilent HPLC with a Bio-Rad Aminex 87-H column with a cation guard. The samples were injected in 5 μ L volumes and run with 0.6 mL/min sterile filtered water at 65°C. The samples were measured with a refractive index detector and analyzed with ChemStation software. This analysis was performed at the Savannah River National Laboratory.

For the fructose and side-by-side species comparisons, a Shimadzu HPLC system consisting of an LC-20AT pump, a CTO-20A, an RID-10A refractive index detector, and a CBM-20A controller with a Bio-Rad Aminex 87-H column held at 80°C was used to measure the concentrations of fructose, glucose, and xylose (unfortunately, this column does not resolve xylulose). The samples were injected in 20 μ L volumes and run with 0.6 mL/min sterile filtered deionized water that had been degassed with helium as the mobile phase. The samples were measured with a refractive index detector and analyzed with Shimadzu software. An Agilent 7890A GC system was used to measure ethanol for the fructose and side-by-side species comparison experiments. Sample of 1 μ L is injected into the inlet, which was at a pressure of 16.2 psi, total flow was 52.5 mL/min, and the injector temperature was at 230°C. The flow through the column (HP-FFAP, Agilent J&W, 25m, 0.32mm, 0.5 μ m) was 0.65 mL/min. The initial temperature of the column was 60°C, and it was held for 1 min and then increased to 120°C at a rate of 3°C/min. The temperature was then increased to 160°C at a rate of 65°C/min, increased to 220°C at 15°C/min and held for 2 min. The hydrogen flow rate was 30 mL/min, the air flow rate was 400 mL/min, and the makeup gas (helium) flow rate was 25 mL/min. ChemStation software was used to analyze the data.

Statistical analysis

The maximum observed cell densities and ethanol concentrations were examined using the statistical analysis software SAS (SAS Institute Inc, Cary, NC). SAS was used to

conduct analysis of variance test ($P \leq 0.05$) via the GLM procedure to determine whether the cell densities and ethanol concentrations were different due to the carbon source (glucose and/or xylose) or due to enzyme addition (xylose isomerase). A similar analysis was conducted for the fructose and xylose isomerase activity and the side-by-side *S. pastorianus* and *S. cerevisiae* experiments.

Genetic profiling

The not transcribed spacer 2 (NTS2) variable region flanked by the 5S and 18S conserved sequences of ribosomal DNA⁴ were polymerase chain reaction (PCR) amplified using the primers AACGGTGCTTTCTGGTAG (forward) and TGTCTTCAACTGCTTT (reverse) to identify the yeast used in this study.³⁵ The PCR product was cloned to the pGEM T vector and sequenced with the forward, reverse, T7, and Sp6 primers. The DNA extraction, amplification, and sequencing reactions were performed by the Clemson University Genomics Institute.

The resultant sequences were assembled using the software Geneious (Biomatters) and analyzed via the BLAST tool provided by NCBI. The experimental sequence showed 99% (1,251/1,257) identity with the *Saccharomyces carlsbergensis* recombinant DNA NTS sequence (GenBank X00468.1) and 99% (1,244/1,257) identity with the *S. cerevisiae* strain BY4848 35S ribosomal DNA intergenic spacer 2 sequence (GenBank DQ130103.1). Based on these DNA results, the yeast species investigated in this study is most closely related to *S. carlsbergensis* (currently named *S. pastorianus*), closely related to *S. cerevisiae*, and is a member of the *Saccharomyces* genus.

Results and Discussion

In preliminary results, *S. pastorianus* were able to grow under very high gravity conditions and produce high concentrations of ethanol (>100 g/L).³⁶ *S. pastorianus* was also able to consume glucose and produce ethanol in medium containing hydrolyzed switch grass and xylose isomerase.³⁶ Additionally, *S. pastorianus* could be cultured on xylose as the sole carbon source with xylose isomerase included in the medium,³⁶ similar to the cell growth observed with 0.5 g/L glucose and 40 and 80 g/L xylose (Figure 1A). Based on these observations, it was determined that *S. pastorianus* could be used to produce ethanol at high levels from mixed sugars and was a viable candidate organism for economically feasible cellulosic-derived ethanol when cultured with xylose isomerase.

Glucose and xylose fermentation with xylose isomerase

To quantify the enhanced ethanol production by *S. pastorianus* due to xylose isomerase, growth with and without xylose isomerase in mixed sugars was examined. The glucose concentrations investigated were 0.5, 80, and 160 g/L; the xylose concentrations were 0, 40, and 80 g/L; and the xylose isomerase additions were 0 and 5 g/L. The glucose and xylose concentrations represent typical values obtained for hydrolyzed switch grass (80 g/L glucose and 40 g/L xylose).¹ A solution mimicking concentrated hydrolyzed switch grass (160 g/L glucose and 80 g/L xylose) was also examined to provide sufficient sugars for higher ethanol concentrations. Final ethanol concentrations greater than 70 g/L

are considered necessary for economic feasibility,^{2,37,38} which requires a minimum of 140 g/L sugars be consumed with theoretical conversion.³¹ The cultures lacking one or both carbon sources provided controls and allowed for the determination of interaction effects. Triplicate cultures were investigated for all conditions, and all combinations were examined.

As expected, all cultures provided with significant amounts of glucose or xylose grew well. Cell growth in the cultures without significant amounts of glucose or xylose (Figure 1A) occurred because of the basal level of glucose (0.5 g/L) in the medium (due to the 1% rich medium addition) and the organic material provided by the rich medium components (0.3 g/L); the cultures with xylose isomerase had better growth. In Figure 1B, growth on 80 g/L glucose and the three levels of xylose with and without xylose isomerase are shown. As can be observed, the cultures without xylose isomerase had the lower growth rates and final cell densities, as indicated by the clear separation of the open symbols (no xylose isomerase) and closed symbols (xylose isomerase). In Figure 1C, growth on 160 g/L glucose and the three levels of xylose with and without xylose isomerase are shown. Again, it is clear that the cultures with the xylose isomerase addition have better growth profiles (closed symbols) compared with the cultures without xylose isomerase (open symbols). Statistical analysis of the maximum cell densities showed that the glucose and xylose isomerase significantly affected the growth of *S. pastorianus* ($P \leq 0.05$), however, xylose did not ($P \geq 0.05$). Improved growth kinetics due to xylose isomerase has not been previously reported, as the initial cell densities used by other researchers were high, such that growth was likely not significant.^{18,19,29}

Cell growth is important to ethanol production, as many researchers have noted,³¹ but ultimately, the ethanol production rate and yields are the final criterion for a successful process. Figure 1 also shows the ethanol concentration profiles for the glucose, xylose, and xylose isomerase cultures grouped by the glucose concentration in the medium. In Figure 1D, the ethanol concentration profiles are shown for the *S. pastorianus* cultures with 0.5 g/L glucose (due to the 1% rich medium addition) on a 0–10 g/L ethanol scale to better visualize the ethanol profiles. The cultures with xylose isomerase and 40 and 80 g/L xylose did result in detectable ethanol levels; whereas the cultures with 0 g/L xylose had no detectable ethanol. Additionally, the cultures without xylose isomerase had no production of ethanol at any of the xylose concentrations.

In Figure 1E, the ethanol concentration profiles are shown for the cultures with 80 g/L glucose and the three levels of xylose, with and without xylose isomerase. The maximum ethanol concentrations were approximately 36 g/L for all of these cultures; however, the cultures with xylose isomerase had faster ethanol production rates. In Figure 1F, the ethanol concentration profiles for the cultures with 160 g/L glucose and the three levels of xylose, with and without xylose isomerase are shown. The maximum ethanol concentrations were over 70 g/L for the cultures with xylose isomerase and slightly lower for the cultures without xylose isomerase; however, note the ethanol production rate is higher for the cultures with xylose isomerase.

The statistical analysis of the maximum ethanol concentration results indicates that ethanol production was strongly increased by glucose, as expected ($P \leq 0.05$). Additionally, the xylose isomerase also statistically affected ethanol

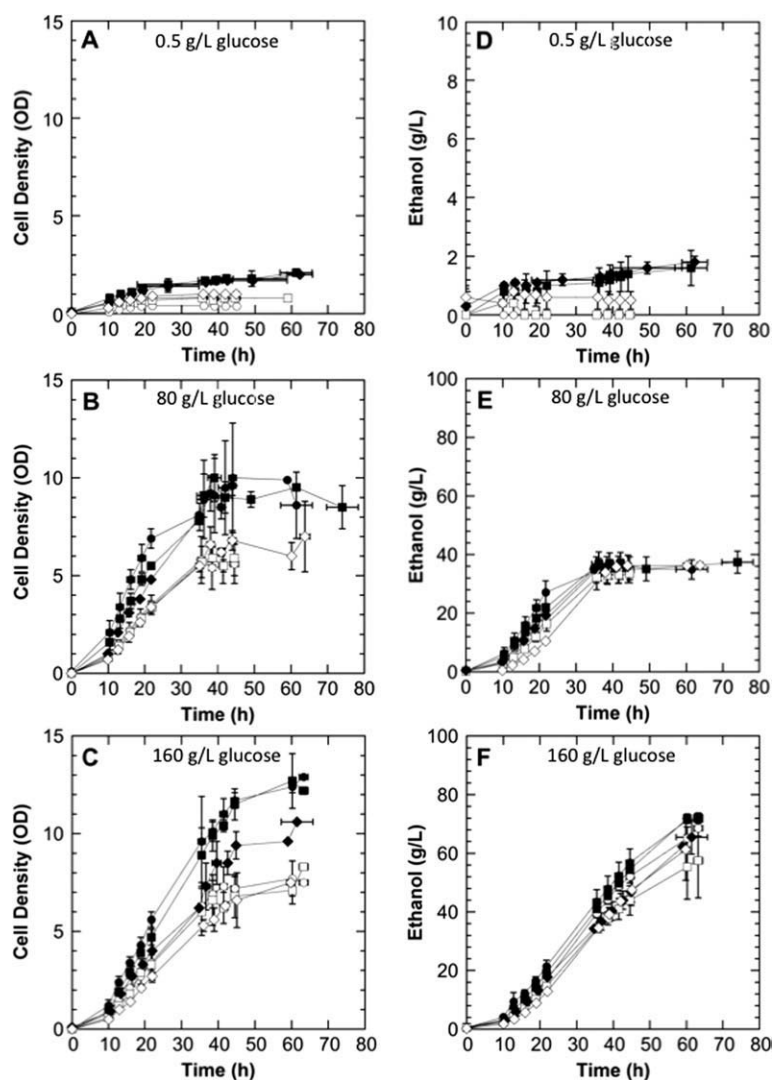


Figure 1. Growth and ethanol concentration profiles for *S. pastorianus* cultured in minimal medium with varying glucose, xylose, and xylose isomerase levels.

The growth profiles are shown in panels (A), (B), and (C). The ethanol concentration profiles are shown in panels (D), (E), and (F). The medium contained 0.5 g/L glucose (A, D), 80 g/L glucose (B, E), or 160 g/L glucose (C, F). Xylose isomerase was either added to the medium at 5 g/L (●, ■, ◆) or not present (○, □, ◇). The xylose concentrations were 0 g/L (●, ○), 40 g/L (■, □), or 80 g/L (◆, ◇). Error bars represent 95% confidence intervals.

production ($P \leq 0.05$); however, xylose did not significantly affect the maximum ethanol concentration ($P \geq 0.05$). Comparisons between the cultures with and without xylose isomerase indicate that xylose isomerase increased ethanol concentration by 12% for the cultures grown on 160 g/L glucose ($P \leq 0.05$); however, xylose isomerase did not result in a statistically significant increase in ethanol when only 80 g/L glucose was used ($P \geq 0.05$). It appears that the xylose isomerase enhances cellular growth rates and increased the overall ethanol yield from the total sugars when high sugar concentrations were used. These results were unexpected, as it was not anticipated that xylose isomerase would be a significant factor for cell growth and ethanol production independent of the xylose concentration.

Previous work using xylose isomerase to produce ethanol from xylose demonstrated that *S. cerevisiae* could convert the xylulose to ethanol, although at a lower rate than obtained for glucose or fructose,¹⁸ that is, 1 h for complete conversion of 60 g/L glucose or fructose compared with 6 h for 60 g/L xylose. However, Chiang et al.¹⁸ converted the

xylose to xylulose in a separate reaction, which would increase the cost of the ethanol significantly. Realizing that preconversion was a cost prohibitive step, Gong et al.¹⁹ continued this work and examined simultaneous conversion and fermentation. Yuan et al.²⁹ took this work further and immobilized the xylose isomerase in a bilayer system to increase the equilibrium conversion of the xylose isomerase by controlling pH to optimal conditions. Table 1 summarizes these previous xylose isomerase results and compares these results to the current work. All these studies indicate that the xylulose uptake by yeast is feasible; however, the bilayer immobilization may not be economical feasible for fuel ethanol due to the high startup costs. Additionally, it is apparent that the fast ethanol production rates observed by Yuan et al.²⁹ are due to high nutrient loading, which when fully accounted in the overall yields, significantly decreases the ethanol yields from the total nutrients used.

To better understand the kinetics of the xylose to xylulose reaction in the mixed sugar cultures of this study, the xylulose profiles were examined in more detail and are shown in

Table 1. Comparison of Ethanol Production Rates and Yields for *S. pastorianus* and *S. cerevisiae* on Biomass Sugars*

Study	This Study	Chiang et al. ¹⁸	Gong et al. ¹⁹	Yuan et al. ²⁹	Almedia et al. ⁴⁴	Bera et al. ²³	Guo et al. ⁴³	Ha et al. ²⁶
Organism	<i>S. pastorianus</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	Recombinant <i>S. cerevisiae</i>	Recombinant <i>S. cerevisiae</i>	Recombinant <i>S. cerevisiae</i>	Recombinant <i>S. cerevisiae</i>
Xylose isomerase	Immobilized	Xylose preconverted	Xylose preconverted	Immobilized	Immobilized	Immobilized	Immobilized	Immobilized
Xylose isomerase Used (g/L)	5	NA	NA	100	18	NA	NA	NA
Inputs								
Total nutrients (g/L) [†]	200	210	41	151	589	110	81	200
Glucose (g/L)	160				130			
Xylose (g/L)	40	60	20	120	50	70	50	60
Xylulose (g/L)								
Cellobiose (g/L)								
Rich components (g/L)	0.3		11	11	9	30	30	100
Biomass (g/L)	0.025	75	5	10	200	4.76	0.25	30
Outputs								
Ethanol (g/L)	70	12.5	12	18	70	27	25	60
Fermentation time (h)	65	6	45	10	30	25	28	72
Biomass (g/L)	6	NR	NR	NR	NR	NR	3.5	17
Overall								
Ethanol production rate (g/L h)	1.08	2.08	0.27	1.80	2.33	1.08	0.89	0.83
Yield from total nutrients (g/g)	0.35	0.06	0.29	0.12	0.12	0.25	0.31	0.30
Yield excluding biomass (g/g) [‡]	0.35	0.21	0.39	0.14	0.37	0.27	0.31	0.32

* Exogenous xylose isomerase with native species or recombinant species data is shown. [†]Total nutrients is the sum of the sugars and rich components added to the fermentation plus two times the biomass inoculum. [‡] (Nutrient inputs – biomass input) = the sum of the sugars and rich media components. NR, not reported; NA, not applicable.

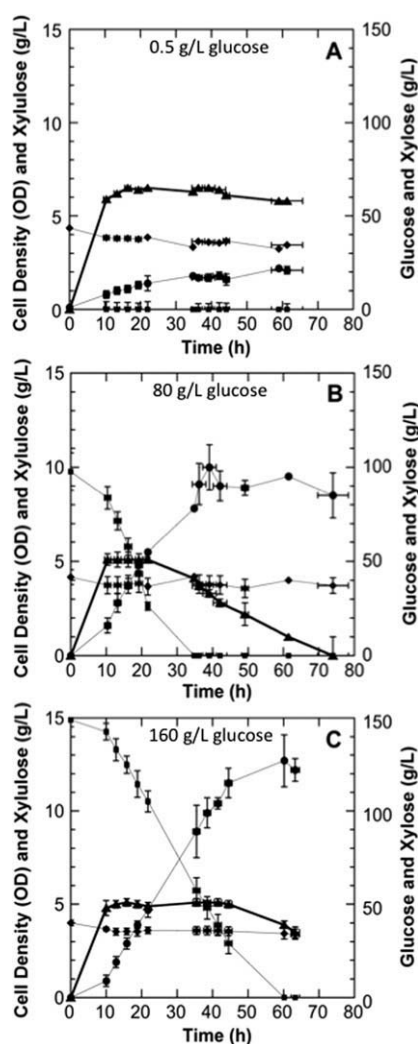


Figure 2. Growth, glucose, xylose, and xylulose profiles for *S. pastorianus* cultured in minimal medium containing 40 g/L xylose, 5 g/L xylose isomerase, and varying glucose concentrations.

The glucose levels were 0.5 g/L (A), 80 g/L (B), or 160 g/L (C). Glucose (■), xylose (◆), xylulose (▲), and cell density (●) data are shown grouped by glucose concentration. Error bars represent 95% confidence intervals.

Figure 2. The cultures with 40 g/L xylose for the three different glucose concentrations with xylose isomerase are shown in detail in Figure 2. The concentration profiles clearly show that equilibrium was reached between xylose and xylulose in the presence of three different glucose concentrations. The xylulose equilibrium concentration was approximately 5 g/L for the cultures with 80 and 160 g/L glucose and 7 g/L for the cultures with 0.5 g/L glucose added. This represents an equilibrium conversion of approximately 12 and 17% xylose, respectively. For the 80 g/L glucose cultures, the xylulose was consumed at a faster rate than the enzyme conversion rate, once glucose was consumed, as shown by the decreasing xylulose concentrations between 20 and 35 h. For the 160 g/L glucose cultures, glucose was completely consumed between 45 and 60 h, thus xylulose was only slightly consumed between 45 and 60 h. Due to a limited number of cells in the 0.5 g/L glucose cultures, xylulose was only consumed very slowly after reaching the equilibrium conversion of 17%.

The 12 to 17% equilibrium conversions for xylose to xylulose in the culture medium is lower than the reported 20% equilibrium conversion under optimal xylose isomerase conversion conditions (70°C, pH 7.0)¹⁸; however, it is significant enough to allow for xylulose to build up in the cultures. The concentration profiles confirmed that xylose was converted to xylulose until the system reached equilibrium (within 10 h). Once the cells consumed the initial glucose in the media, the cells could consume the xylulose. Yuan et al.²⁹ report similar equilibrium conversion findings with approximately 16% conversion of xylose to xylulose during simultaneous isomerization and fermentation (within 10 h). In their setup, a bilayer xylose isomerase pellet was used that contained borax and urease to increase the pH near the xylose isomerase as a means to increase the equilibrium conversion.^{29,30} It might be possible to increase the observed equilibrium of this study by the addition of borax in the culture media.

The results of these growth experiments demonstrated that xylose isomerase significantly impacted the growth of *S. pastorianus*. The cultures with both glucose and xylose isomerase had significantly higher observed cell densities compared with the cultures without xylose isomerase (approximately 30% more cells). Xylose isomerase is also a glucose isomerase and thus can convert glucose to fructose, and both of these isomerization reactions are reversible. Fructose can also be converted efficiently to ethanol,¹⁸ thus the effect of fructose on growth and ethanol production were examined to determine the role of fructose in the enhanced growth rates and ethanol production observed in the glucose/xylose fermentations.

Fructose fermentation with xylose isomerase

S. pastorianus was grown on fructose with and without xylose isomerase in the standard minimal medium supplemented with 1% rich medium without glucose to determine if whether the cometabolism of glucose and fructose could account for the higher cell densities and improved ethanol production. Additionally, xylose was not added to the media. Unlike *S. cerevisiae*, *S. pastorianus* has separate glucose and fructose transporters.¹⁴ As controls, *S. pastorianus* were cultured in medium with glucose and xylose isomerase in parallel. Figure 3 shows the growth profiles, maximum cell densities, and ethanol production levels for *S. pastorianus* grown on 80 g/L fructose and 160 g/L fructose, respectively, with and without xylose isomerase. The glucose controls are also shown in Figure 3. The initial growth rates for the fructose and glucose cultures containing xylose isomerase were higher than the cultures without enzyme (Figures 3A,B), which indicates that xylose isomerase significantly affected the growth rate of *S. pastorianus* regardless of the original carbon source (glucose or fructose) and in the absence of xylose. Statistical analysis of the maximum cell density values for the cultures grown on fructose and glucose showed that fructose, glucose, and xylose isomerase were all significant factors ($P \leq 0.05$). Further analysis showed that xylose isomerase enhanced the maximum cell density of cultures grown on 80 and 160 g/L glucose or fructose by 30% and 22%, respectively.

The ethanol concentration profiles for fructose and glucose with xylose isomerase are shown in Figures 3C,D. As expected, ethanol concentrations were lower for the 80 g/L cultures compared with the 160 g/L cultures for both glucose

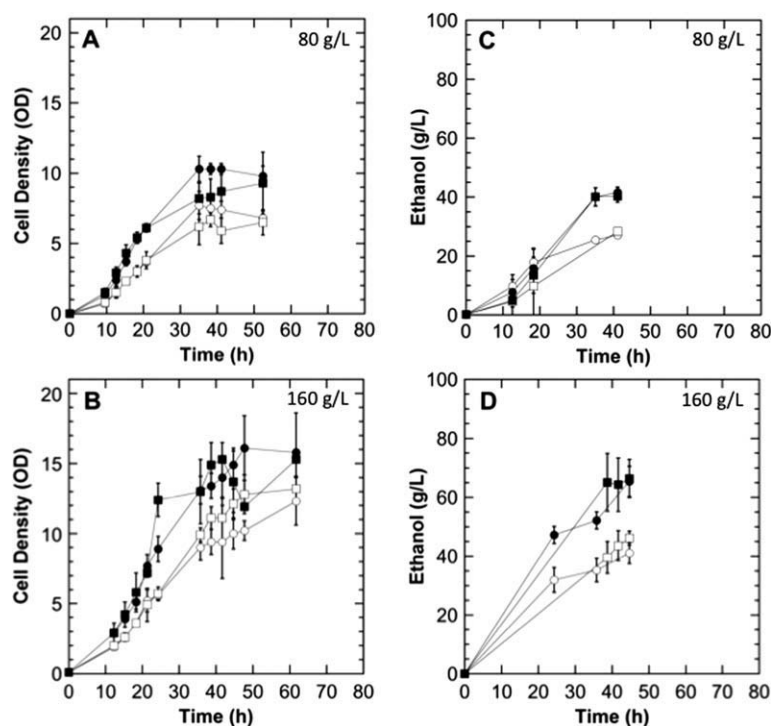


Figure 3. Growth and ethanol concentration profiles for *S. pastorianus* cultured in minimal medium with fructose or glucose as the sole carbon source.

The medium contained 80 g/L (A, C) or 160 g/L (B, D) fructose (■, □) or glucose (●, ○), respectively. Xylose isomerase was either added to the medium at 5 g/L (■, ●) or not present (□, ○). Error bars represent 95% confidence intervals.

and fructose. Statistical analysis of the maximum ethanol concentration for the cultures grown on fructose and glucose showed that fructose, glucose, and xylose isomerase were all significant factors ($P \leq 0.05$). Ethanol levels obtained from the 80 g/L fructose and glucose cultures with xylose isomerase were approximately 44 g/L ethanol, while without xylose isomerase only approximately 30 g/L ethanol was obtained. These values represented a conversion of sugar to ethanol of 55% and 38%, respectively (Figure 3C).

For the 160 g/L fructose and glucose cultures with and without xylose isomerase, ethanol concentrations were also significantly different due to the addition of xylose isomerase. The 160 g/L glucose and fructose cultures with xylose isomerase produced approximately 70 g/L ethanol at 45 h, which represented a 44% conversion. In comparison, the 160 g/L sugar cultures without xylose isomerase produced only approximately 45 g/L ethanol at 45 h. Based on the sugar consumption profiles previously observed (Figures 1 and 2), and the cell density profiles of these cultures, it appears the observed ethanol concentration may not be representative of the maximum ethanol concentrations for these conditions. The mechanism behind the increased conversion of sugar to ethanol in the presence of xylose isomerase is hypothesized to be dual metabolic overflow metabolism due to the presence of the two sugars in the media. Confounding analysis of this hypothesis is that the fructose uptake rate is faster than the enzyme conversion rate, as no fructose was detected in the glucose cultures, and similarly no glucose was detected in the fructose cultures (data not shown). However, these results are consistent with the dual metabolic overflow hypothesis.

The positive effect of xylose isomerase on growth and ethanol production, even in the absence of xylose, suggests that the glucose isomerase may be an important factor. Alter-

natively, the immobilization agent in the xylose isomerase matrix material may be contributing substrate to the cells to produce ethanol. The reusability of the enzyme, no apparent mass change of the immobilized enzyme during the fermentation, and the longevity and stability of this enzyme matrix in the high fructose corn syrup industry, make it unlikely that significant carbon redirection is occurring; however, it is possible that the immobilization agent is providing an unknown micronutrient. Comparing growth and ethanol production on the matrix material alone would be one method to determine the contributing factors of the enzyme and the matrix.

Xylose isomerase activity

To address the possibility that the immobilization agent is leaching into the culture media and providing nutrients for ethanol production, and cultures with inactive enzyme on the matrix material were investigated. The xylose isomerase was supplied immobilized on a support matrix that is rigid and darkly colored. As the manufacturer would not describe the support matrix or the immobilization process, the enzyme was inactivated on the immobilization matrix to provide a matrix material with enzyme activity, that is, inactive enzyme. For these experiments, the enzyme on the support was deactivated by autoclaving on a liquid cycle for 15 min at 121°C. The enzyme deactivation was confirmed by incubating enzyme in the culture medium containing 160 g/L glucose and 40 g/L xylose without cells. Figure 4 shows the growth, glucose, xylose, xylulose, and ethanol profiles for cultures with the inactive xylose isomerase compared with active enzyme and no enzyme controls. Statistical analysis of the final cell densities indicated that the final cell densities for all the conditions were significantly different ($P \leq 0.05$).

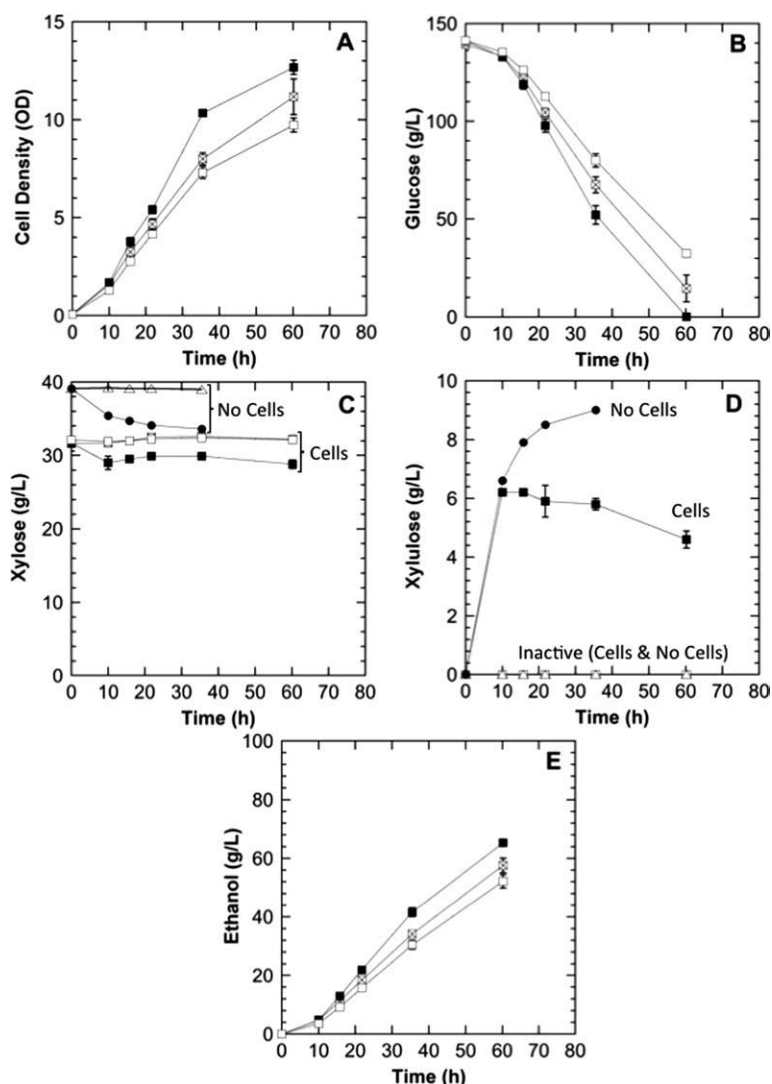


Figure 4. Growth, glucose, xylose, xylulose, and ethanol concentration profiles for *S. pastorianus* cultured in minimal medium containing glucose, xylose, and varying enzyme activity.

The inactive enzyme was obtained by autoclaving the enzyme. Cell density (A), glucose (B), xylose (C), xylulose (D), and ethanol (E) profiles are shown for the active enzyme (■), inactive enzyme (□), or no enzyme (△). Additionally, the xylose (C) and xylulose (D) profiles are shown for media without cells for the active enzyme (●) and inactive enzyme (△). Error bars represent 95% confidence intervals.

The glucose profiles (Figure 4B) mirror the growth profiles, indicating that fastest glucose consumption is the cause of the higher growth rates. The xylose (Figure 4C) and the xylulose (Figure 4D) profiles confirm that the inactive enzyme did not convert xylose to xylulose, with and without cells. The ethanol concentration profiles (Figure 4E) for the three cultures show that ethanol was produced in all culture conditions, as expected. Analysis of the maximum ethanol concentrations confirmed that the cultures containing active xylose isomerase were significantly higher ($P \leq 0.05$) than the cultures with inactive xylose isomerase, and the inactive enzyme cultures had higher ethanol concentrations than the no enzyme cultures. Taken together, these results indicate that the xylose isomerase enzyme and the support matrix both contribute to the enhanced growth and ethanol production.

Side-by-side comparisons of *S. pastorianus* and *S. cerevisiae*

To determine whether the enhanced ethanol productivity due to the immobilized xylose isomerase was unique to

S. pastorianus, side-by-side experiments were conducted comparing *S. pastorianus* and *S. cerevisiae*. The culture temperature was selected to be favorable to *S. cerevisiae* (30°C). The growth profiles for both species in medium containing either 160 g/L glucose and 40 g/L xylose or 160 and 80 g/L xylose (with and without xylose isomerase) are shown in Figures 5A,B, respectively. The presence of xylose isomerase resulted in higher growth rates for both species in both media. The *S. pastorianus* under both conditions had higher growth rates compared with *S. cerevisiae*. The xylose concentration did not significantly affect the growth characteristics. The glucose and xylose profiles for both the species in medium containing either 160 g/L glucose and 40 g/L xylose or 160 and 80 g/L xylose (with and without xylose isomerase) are shown in Figures 5C,D, respectively. The glucose profiles for both yeast species in the presence of xylose isomerase were similar; however, in the cultures without xylose isomerase, the *S. pastorianus* cultures consumed glucose faster than the *S. cerevisiae* cultures. The effect of the xylose concentration on the glucose consumption rates was not significant. The xylose profiles demonstrate that the

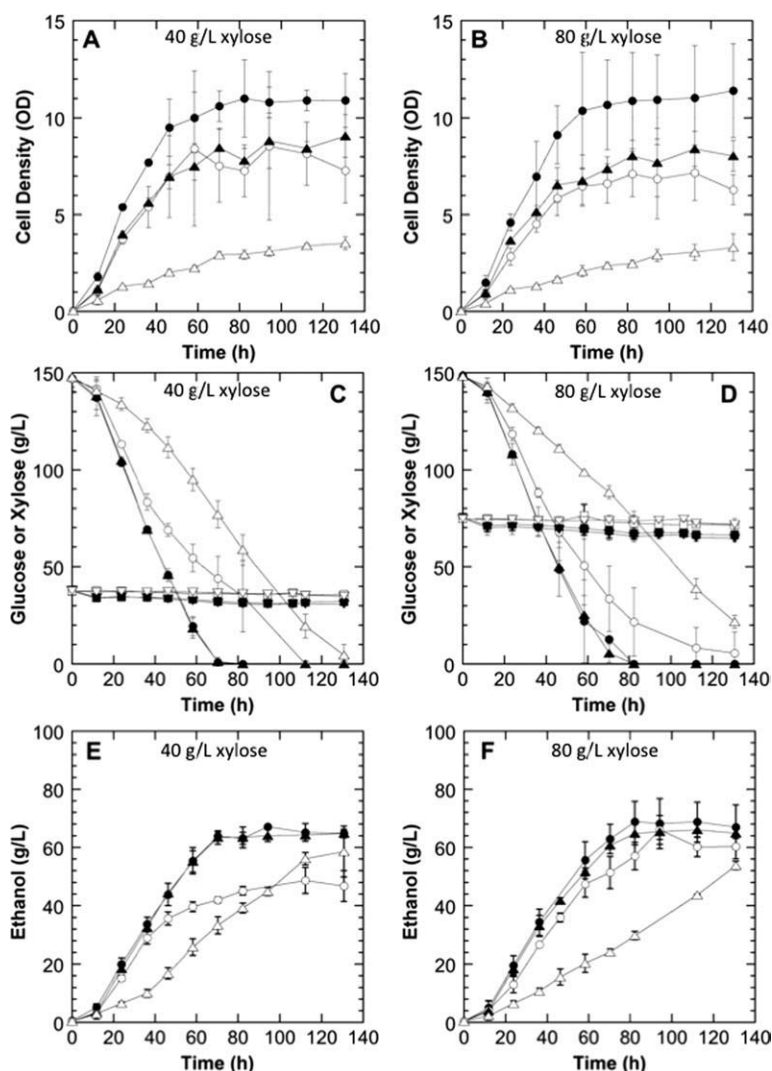


Figure 5. Growth, glucose, and ethanol concentration profiles for *S. pastorianus* and *S. cerevisiae* cultured in minimal medium with 160 g/L glucose and varying xylose and xylose isomerase levels.

The growth profiles are shown in panels (A) and (B). The glucose (●, ○, ▲, △) and xylose (■, □, ▼, ▽) profiles are shown in panels (C) and (D). The ethanol concentration profiles are shown in panels (E) and (F). The medium contained 40 g/L xylose (A, C, E) or 80 g/L xylose (B, C, F). *S. pastorianus* (●, ○, ■, □) and *S. cerevisiae* (▲, △, ▼, ▽) are shown with 5 g/L xylose isomerase (●, ■, ▲, ▼) or without xylose isomerase (○, □, △, ▽). Error bars represent 95% confidence intervals.

xylose isomerase was functional, as the cultures with xylose isomerase had lower xylose levels throughout. There was no significant difference in the xylose profiles between the species, which would indicate that xylulose profiles were similar. The ethanol concentration profiles for both species in medium containing either 160 g/L glucose and 40 g/L xylose or 160 and 80 g/L xylose (with and without xylose isomerase) are shown in Figures 5E,F, respectively. The presence of xylose isomerase resulted in higher ethanol production rates for both species in both media and with similar profiles. The effect of xylose isomerase to improve the ethanol production rate was more pronounced for *S. cerevisiae* although significant for both the species. The xylose concentration did not significantly affect the ethanol production profiles for *S. cerevisiae*; however, *S. pastorianus* ethanol production was faster at the higher xylose concentration without xylose isomerase. Xylose isomerase increased ethanol production rates in both yeast species due to faster glucose consumption rates. Interestingly, the biomass generated for *S. pastorianus* was higher than that for *S. cerevisiae* despite similar ethanol

profiles. *S. pastorianus* appears to have equal or higher tolerance to xylose than *S. cerevisiae*. Additionally, the higher biomass production of *S. pastorianus* may improve process economics, as it is common to sell the biomass from bioethanol processes.²⁸

Numerous research groups have approached the production of ethanol in *S. cerevisiae* from cellulosic-derived xylose by introducing genes into this organism to allow for xylose metabolism.^{20,21,39,40} These recombinant *S. cerevisiae* approaches have been preferred to using organisms that naturally metabolize xylose, in part due to natural high ethanol tolerance, high ethanol productivity, and high ethanol yields of *S. cerevisiae*.^{25,41} Early studies focused on the addition of xylose isomerase genes; however, it was soon recognized the redox balance in the recombinant cells was compromised and xylose transport was slow, which negatively impacted cell growth and ethanol production.⁴² Recent studies have worked to overcome these issues by introducing genes, such as xylose reductase, xylitol dehydrogenase, xylulokinase, and hexose transporters.^{23,43,44} Additionally, genes for cellobiose

transport and metabolism have been inserted into *S. cerevisiae*, in addition to the xylose metabolism genes to circumvent the diauxic growth that is observed when glucose and xylose are used as the carbon sources for growth and ethanol product in native and recombinant *S. cerevisiae*. Comparisons of these recent recombinant *S. cerevisiae* approaches to the present work are shown in Table 1. The *S. pastorianus* and *S. cerevisiae* cultures with xylose isomerase are able to produce up to 70 g/L ethanol from 160 g/L glucose, 40 g/L xylose, 0.1 g/L yeast extract, and 0.2 g/L peptone in 65 h starting with 0.05 OD cultures (Figures 1–5). This results in an ethanol production rate of 1.08 g ethanol per liter hour, which is comparable with the best recombinant *S. cerevisiae* values obtained by Bera et al.²³ Also, important to the economics of cellulosic-derived ethanol is the yield of ethanol from the supplied nutrients. In this work, the overall ethanol yield was 0.35 g ethanol per gram of supplied nutrients. Again, this ethanol yield value is comparable (or higher) with the best ethanol yields reported for recombinant *S. cerevisiae*.⁴³ Although the xylose has not been effectively converted to ethanol in the current process, the process was improved due to xylose isomerase, and should be further investigated. Additionally, it may be possible to combine immobilized xylose isomerase with recombinant *S. cerevisiae* fermentations to decrease the culture times, which would increase ethanol productivity.

Conclusions

Xylose isomerase increases cell growth and ethanol productivity for both yeast species *S. pastorianus* and *S. cerevisiae* due to enhanced glucose consumption rates. The mechanism by which xylose isomerase improves cell growth and ethanol productivity appears to be related to the conversion of glucose to fructose by the enzyme. Additionally, these results demonstrated that *S. pastorianus* has the potential to convert cellulosic-derived sugars to ethanol at near theoretical conversion levels while obtaining higher biomass concentrations than *S. cerevisiae*. As one income source for bioethanol production is the cell mass, the higher *S. pastorianus* biomass has the potential to enhance the overall economics of the process. Additionally, the incorporation of immobilized xylose isomerase may also have positive impacts on recombinant *S. cerevisiae* ethanol yields and productivity.

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