Supplemental Materials

S.1 Improvements to the *E. coli* Fed-Batch Fermentations to Generate ECE

ECE provided two benefits to the *S. pastorianus* ethanol fermentations on hydrolysate. First, the ECE was used to recycle the xylose carbons obtained from the lignocellulosic material. Second, the ECE provided micronutrients to the S. *pastorianus*, since commercial yeast extract or other complex nutrients traditionally added to the media were not provided in this study [1]. As *E. coli* cells are well characterized in the literature and considered tightly regulated [2-4], the chemical composition of *E. coli* is considered to be approximately $\boldsymbol{CH_{1.9}O_{0.5}\,N_{0.24}S_{0.004}}$ [5], where the biomass on a dry basis on when cultured aerobically on xylose is 61% protein, 18% RNA, 6%lipids, and 5% glycogen [6]. And like commercial yeast extract, the heat-killed *E. coli* biomass proteins would provide all amino acids [6]. ECE is not a perfect replacement for commercial yeast extract for S. *pastorianus*, as *S. pastorianus* have higher glucose consumption rates and ethanol productivity on commercial yeast extract compared to ECE (**Figure S1**). However, ECE allowed for higher glucose consumption rates and ethanol productivity than ammonium chloride (**Figure S1**). Additionally, we were unable to find a native yeast species that could consume xylose in a reasonable timeframe to coordinate with the ethanol fermentation duration [7].

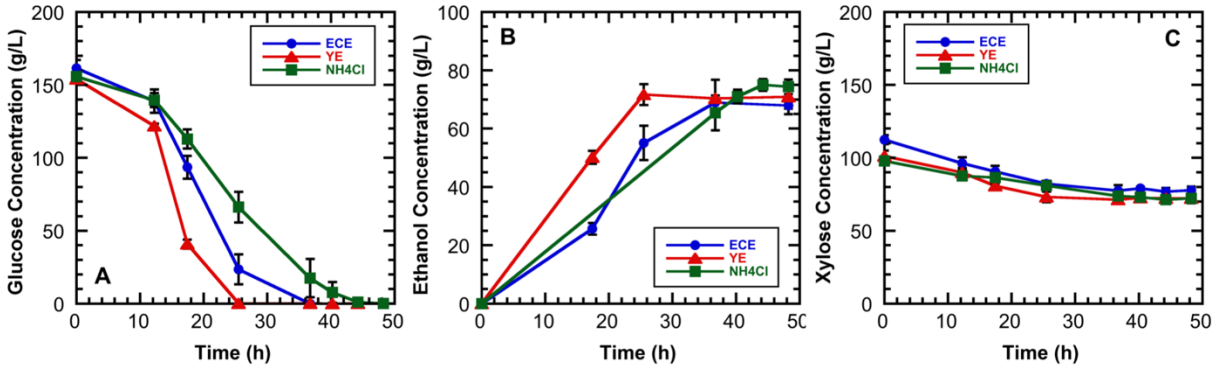

Figure S1: Effect of the nitrogen source on the glucose consumption rate and ethanol productivity for *S. pastorianus* on reagent-grade sugars. A) Glucose consumption, B) ethanol productivity, and C) xylose consumption for *S. pastorianus* cultured with xylose isomerase and either 17 g dcw/L ECE (●), 17 g /L commercial yeast extract (YE) (▲), or 9.0 g/L ammonium chloride (NH4Cl) (■). The final ethanol yields were not statistically different ($p > 0.05$); however, the glucose consumption rate and ethanol productivity were all significantly different ($p \leq 0.05$). Error bars represent the standard deviation.

The *E. coli* fermentation conditions were conducted using exponential feeding with xylose as the sole carbon source to maximize the biomass concentration in the ECE, which would minimize dilution of the ethanol fermentation media. Run 1 was presented previously [8]. Run 1 had an exponential fed rate of 0.15 h^{-1}. Run 2 was a repeat of Run 1 and demonstrated the reproducibility of the fermentation outcome (**Figure S2**). Periodic dissolved oxygen (DO) checks were used to confirm that the xylose was being consumed as the limiting nutrient. Specifically, the xylose feed was stopped briefly and periodically. If the DO increased rapidly (spiked) when the feed pump was stopped, the culture was considered xylose limited. Both Runs 1 and 2 were determined to be nutrient limited in the fed-batch phase by this method. The final cell densities were approximately 86 optical density (OD) (43 g dcw/L) with a fermentation duration of 30 hours. The yield coefficients for biomass from xylose ($Y_{X/S}$) was 0.35 g/g. ECE used in the present work was from Run 2. Also, this yield coefficient is consistent with the batch work of Gonzales et al. (2017) [6].

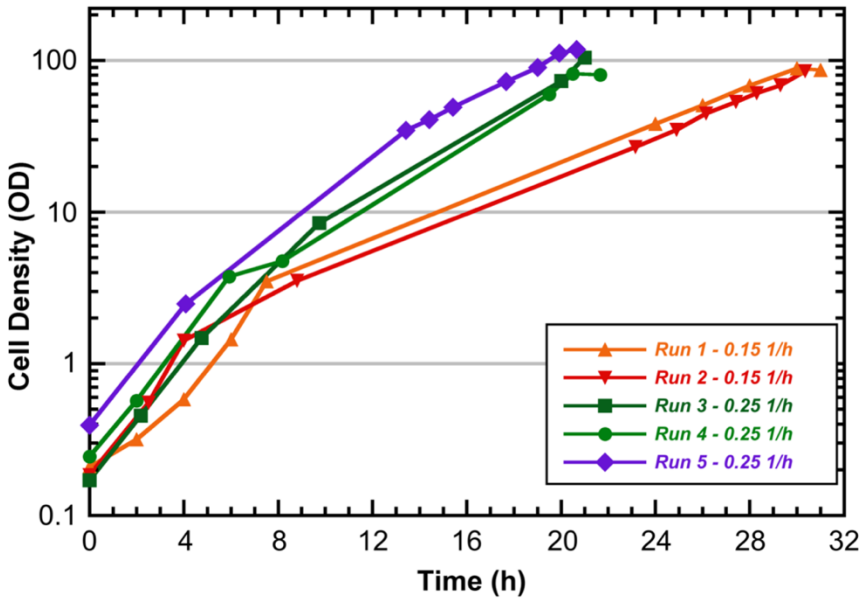

Figure S2: Cell density profiles for *E. coli* fed-batch cultures where xylose was the sole carbon source. Runs 1and 2 had exponential feed rates of 0.15 h^{-1}, while Runs 3 to 5 had exponential feed rates of 0.25 h^{-1}. The batch growth rates were the same for all the cultures. All fermentation data points shown.

Since the batch growth rates of 0.45 h^{-1} are observed on xylose in minimal media, higher feed rates were examined to determine if the productivity and yield could be increased. Based on the glucose feed rate maximums, 0.25 h^{-1} was selected [9,10]. Runs 3 and 4 had exponential feed rates of 0.25 h^{-1} and reached 104 and 88 OD (**Figure S2**), respectively. The biomass yield coefficients from xylose were 0.45 and 0.37 g/g, respectively. Run 4 had a longer starvation period between the batch and fed-batch phases than Run 3, which contributed to the poorer outcome. Interestingly, this appeared to be a critical parameter for optimization when xylose is the sole carbon source. In contrast, *E. coli* cultured on glucose as the limiting substrate are not adversely affected by starvation periods of several hours. In fact, some published *E. coli* fed-batch protocols use this starvation period to reduce the acetate levels after the batch phase [11,12]. In order to investigate the effects of the starvation phase duration, Run 5 was conducted using the same exponential fed profiles as Runs 3 and 4 (0.25 h^{-1}); however, the feed pump was programmed to start 1-hour earlier, to avoid any starvation phase. The final cell densities was 118 OD (for Runs 5.

To further investigate the apparent nutrient-limitation due to dissolved oxygen (DO) spikes when the feed pump was stopped, the xylose concentrations were quantified off-line for Runs 5. Based on the xylose concentrations at the time of the DO spikes, the cultures were not xylose limited. In fact, the xylose concentrations were significantly greater than 1 g/L. Nonetheless, when the feed pump was restarted, the DO quickly returned to normal, indicating the return of exponential growth. These DO responses imply that there was a nutrient limitation. The only other component in the feed solution was magnesium sulfate ($MgSO_4$). These data indicate that Mg^{+2} was the likely limiting nutrient. Further work is needed to confirm the Mg^{+2} limitation hypothesis. **Table S3** summaries the key outcome characteristics of the different runs. Elimination of the Mg limitations could further improve the cell yield from xylose. These multiple *E. coli* fermentations with xylose as the sole carbon source do demonstrate that ECE production can be accomplished in under 24 hours and generate over 50 g dcw/L ECE from xylose. These xylose fermentations would allow the ECE to be generated in tandem, or staged with the *S. pastorianus* ethanol production fermentations.

Table S3: Key characteristic of the fed-batch xylose fermentations. E. *coli* were cultured on minimal medium with xylose as the limiting carbon source with exponential feed.

Run	Exponential Feed Rate (h^{-1})	Final OD	Dry cell weight (g dcw/L)	$Y_{x/s}$ (g/g)	Notes
1	0.15	84	42	--	[8]
2	0.15	86	43	0.35	Repeat of Run 1; Material used in this study
3	0.25	104	52	0.45	1-hour shorter starvation than Run 4
4	0.25	88	44	0.37	Long Starvation
5	0.25	118	59	0.37	Not xylose limited at time of DO spiked; confirmed by off-line xylose quantification

S.2 Development of the fed-batch hydrolysis reaction to generate high gravity sugar concentrations

The sugar concentrations obtained from standard protocols were not sufficient to support high gravity fermentations. Specifically, the enzymatic conversion of the pre-treated sugarcane bagasse to hydrolysate initially followed the protocol described in Jain and Walker [13], except for the substitution of "low activity CTec2" instead of Accelerase® 1500. The CTec2 and HTec2 were labeled "low activity" due to personnel communications with the Novozyme representative responsible for donating the material to Clemson (Kurt Creamer, August to October 2014 emails). The CTec2 enzyme provided to Clemson was not commercial grade. **Table S2** lists the amounts or concentration of a component included in the standard hydrolysis batch reaction. The hydrolysis reaction took 24 hours at 50°C and 200 rpm. Solids were removed by centrifugation at 3000 *g* for 10 min and discarded. The final supernatant/liquid volumes recovered was ~17 mL. The resulting hydrolysate contained between 65 to 78 g/L glucose and 20 to 25 g/L xylose. Therefore, methods were examined to decrease the water content in the reaction mixture.

Table S2: Components for standard enzymatic hydrolysis of pre-treated sugarcane bagasse to generate sugarcane bagasse hydrolysate. The reactions were conducted at 50°C and 200 rpm for 24 hours.

Component (amount or concentration)	Quantity in Batch
Pre-treated bagasse (total mass, g)	18
Enzyme (mL)	2.7
Citrate buffer, pH 4.8 (mL)	2.4
Citrate buffer concentration, pH 4.8 (M)	1.0

The first source of water examined in the reaction mixture was the enzyme itself. There were four enzymes readily available to screen: CTec2 (low activity), HTec2 (low activity), Accelerase® 1500, and Alternafuel CMAX, using the standard conditions [13,14]. The pre-treated material loadings were: 16% dry weight per volume and equal volumes of enzyme. The glucose released for each enzyme is shown in **Figure S3A.** The CTec2 was able to release more glucose from the pre-treated sugarcane bagasse than the other three enzymes. Further work reported by Jain et al. (2016), also with sugarcane bagasse, confirmed that CTec2 worked best at releasing the glucose from glucan and xylose from the xylan [14]. Therefore, all further optimization work used the CTec2 enzyme.

The second source of water examined in the reaction mixture was the volume (amount) of enzyme used, as CTec2 was provides as a liquid. The enzymatic amounts examined were 0.15 to 2.4 mL CTec2, where all other amounts added match the values in **Table S2** [14]. The final glucose concentrations are shown with respect to the CTec2 addition (**Figure S3B)**. The 1.2 mL CTec2 addition resulted in the same glucose concentration as the 2.4 mL addition. Noteworthy, the times for the 0.3 and 0.6 CTec2 mL

additions were much longer than the 1.2 and 2.4 mL additions, in part due to poorer liquidification. And, for the 0.15 mL CTec2 addition, the reaction mixture never liquidified. Further work used the 1.2 mL CTec2 amount and watch for liquidification as a key indicator.

The third source of water in the reaction mixture was the citrate buffer. The citrate buffer volume (amount) was varied from 1.0 to 2.9 mL using the 1.2 mL CTec2 volume. The pre-treatment material loadings for the 1.0 M citrate buffer enzyme reactions were higher than the previous two cases at 23% pre-treated material loaded. In this case, all final glucose concentrations were higher than previous reaction conditions (**Figure S3C**). Nonetheless, based on these data alone, the reaction was limited in part by pH control. Since a 1.0 M citrate buffer is not as saturated solution, a more concentrated citrate buffer (2.0 M citrate buffer) was examined. A 1.5 mL buffer volume with 2.0 M citrate was able to controlled pH well, whereas the pH drop significantly in the cases where less citrate buffer was used (**Figure S3D**).

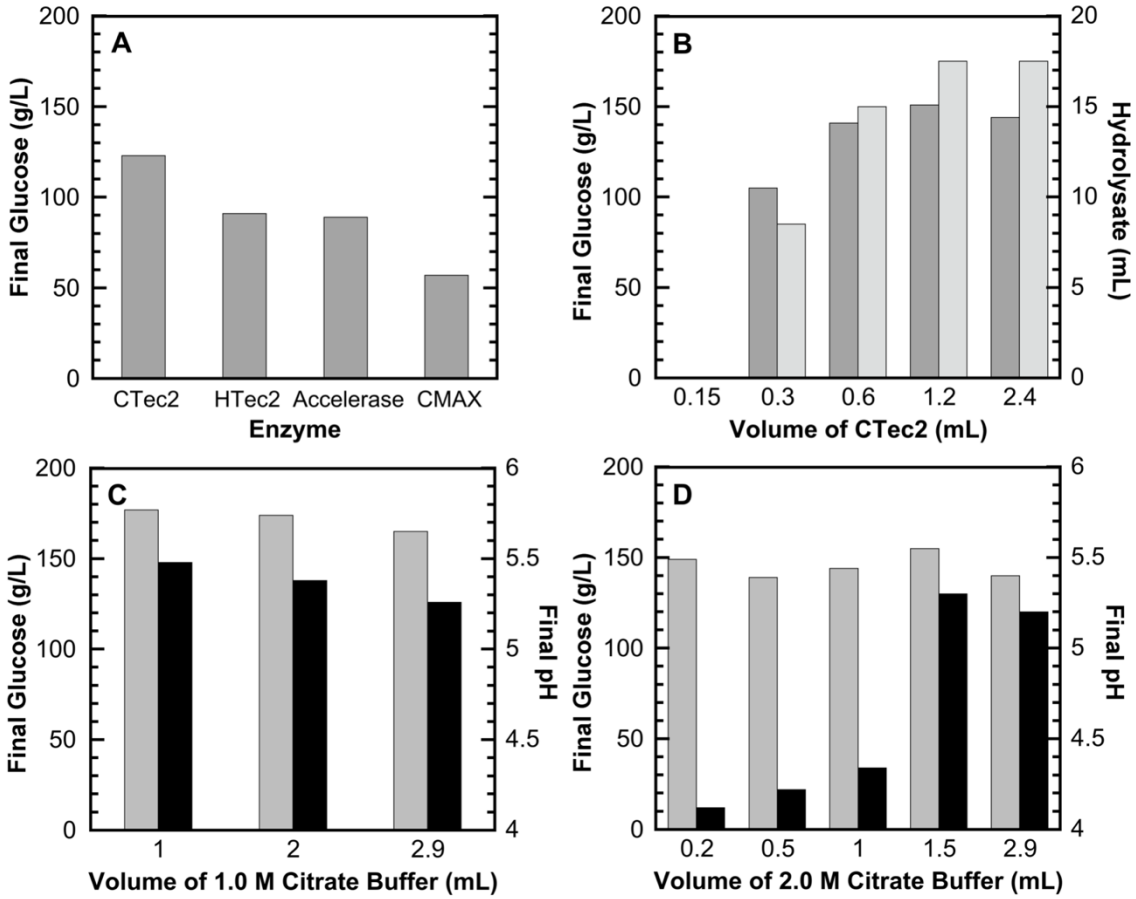

Figure S3. Effects of the enzyme on the hydrolysis reaction final glucose outcomes. (**A)** The hydrolysis reactions were screened using several different enzymes. (**B)** The volume addition of the CTec2 enzyme was decreased from the standard protocol to determine the effect on the sugar yield (dark grey bars) in the hydrolysate. Light grey bars represent the final volume. (**C)** The volume of the buffer was varied to determine the effect on pH versus the sugar yield. (**D)** A concentrated buffer was used to determine the effect of its volume on the sugar yield. The grey bars represent the final glucose concentration and the black bars represent the final pH in panels **(C)** and **(D).** All of these screening experiments were singletons.

In parallel, it was determined a pre-treated moisture content of 76% would enable a 25% loading, which would allow for 180 g/L glucose in the hydrolysate; however, the batch reaction process had no visible liquid with a 21% pre-treatment loading. Consequently, a simple fed-batch approach was examined to provide initial liquefaction and pH control throughout the enzyme reaction process. Based on the optimization studies, a larger-scale process was developed. This fed-batch process used an initial 90 to 91 g bagasse, then added per addition 90 to 91 g bagasse for a of approximately 270 g bagasse per reaction. The moisture content of these additions was between 68 and 70% moisture. The amount of enzyme used was 12 mL per batch reaction with 9.5 mL of 1.5 M citrate buffer at pH 4.8 per reaction. The additional re-treated material was added to the reaction at 24 and 48 hours. Final glucose concentrations between 177 and 226 g/L were obtained (201 g/L glucose and 55 g/L xylose when four reactions were mixed). A second set of three independent runs were conducted of the fed-batch process. When these set of three reactions were mixed together the sugar concentrations were 186 g/L glucose and 50 g/L xylose. As the time interval between the pre-treatment additions was not optimized, the reported 24 hour interval is most likely much longer than necessary. **Table S3** lists the composition of the standard batch reaction [14], the improved batch reaction, and developed fed-batch reaction. All three reaction recipes have been normalized to 100 g total bagasse to assist with direct comparison of the reaction components. Much of the increased glucose concentration in the hydrolysate is due to the elimination of water additions.

Table S3: Enzymatic hydrolysis component for pre-treated sugarcane bagasse. Comparison of the Normalized Standard Batch [14], Normalized Improved Batch, and the Normalized Fed-batch quantities on a 100 g total mass basis.

Component (amount or concentration)	Quantity		
	Normalized Standard Batch	Normalized Improved Batch	Normalized Fed-batch
Pre-treated bagasse (total mass, g)	100	100	100
Enzyme (mL)	15	6.6	4.4
Citrate buffer, pH 4.8 (mL)	13.3	8.3	3.5
Citrate buffer concentration, pH 4.8 (M)	1.0	2.0	1.5
Typical final glucose concentration (g/L)	70	120	200
Typical final xylose concentration (g/L)*	22	44	55

*xylose measurements have significant matrix effects using the YSI (Yellow Sping Instrument) due to the ECE and hydrolysate.

The overall glucose yield in the hydrolysis step for the fed-batch reaction was approximately 67% of the glycan to glucose and 37% of xylan to xylose. The objective for the fed-batch hydrolysis reaction development in this study was to reach high gravity sugar concentrations, and not necessary to optimize the hydrolysis reaction. High gravity sugar concentrations were achieved. Further work, very likely, could improve the yield and productivity of the hydrolysis reaction. The overall bagasse to ethanol yield is approximately 10% (99 g ethanol per 1000 g dry mass bagasse).

S3. Two-Stage Fermentation Process Overview

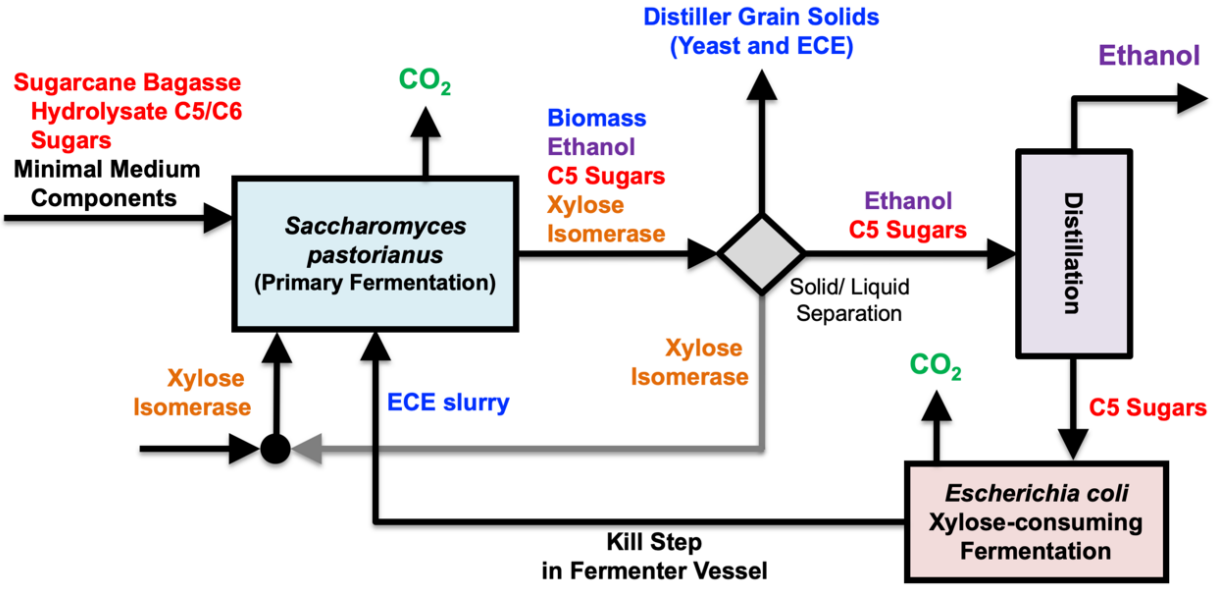

Figure S4: Process diagram for novel two-stage fermentation process to convert glucose and xylose to ethanol by S. *pastorianus*. Hydrolysates, *E. coli* extract (ECE), and xylose isomerase are used by *S. pastorianus* to produce ethanol in the Primary Fermentation step. The *S. pastorianus* biomass and other solids are removed prior to distillation to recover ethanol. The xylose isomerase can be recovered and reused. Xylose recovered from the distillation step is consumed in the *E. coli* fermentation. Killed *E. coli* as ECE is added to the *S. pastorianus* fermentation, and the cycle repeats. Adapted from Gowtham et al., 2014 [8].

S.4 Effect of ECE and Xylose Isomerase amounts on ethanol productivity in reagent-grade sugars

S.4.1 ***Xylose Isomerase Effect on Ethanol Productivity***

To determine the impact of xylose isomerase on ethanol productivity for *S. pastorianus*, cultures were grown on reagent-grade sugars with ECE and with and without xylose isomerase. Duplicate cultures were examined. The glucose, xylose, and ethanol profiles are shown in **Figure S5**, where in the initial sugar concentrations in the fermentation broths were 150 g/L glucose and 75 g/L xylose. The glucose consumption rate was faster for *S. pastorianus* with ECE and xylose isomerase at 5.25 ± 0.07 g glucose/L·h compared to 3.24 ± 0.07 g glucose/L·h without xylose isomerase, which were significantly different ($p \leq 0.05$). The xylose profiles and overall conversions demonstrate that the xylose isomerase was functional in the reagent-grade sugar media, and reached equilibrium values. The ECE causes a matrix effect, which results in an ~25 g/L offset in the xylose values. This offset decreases as the cells utilize the ECE. However, between the culture conditions, xylose conversion was higher for the cultures with xylose isomerase at 37% compared to only 22% without xylose isomerase ($p \leq 0.05$). The ethanol productivity was 2.24 ± 0.04 g ethanol /L·h for the culture with both ECE and xylose isomerase and only 1.20 ± 0.04 g ethanol /L·h for culture with only ECE. The xylose isomerase significantly affected ethanol productivities ($p \leq 0.05$); however, did not significant effect the final ethanol concentration ($p = 0.0776$).

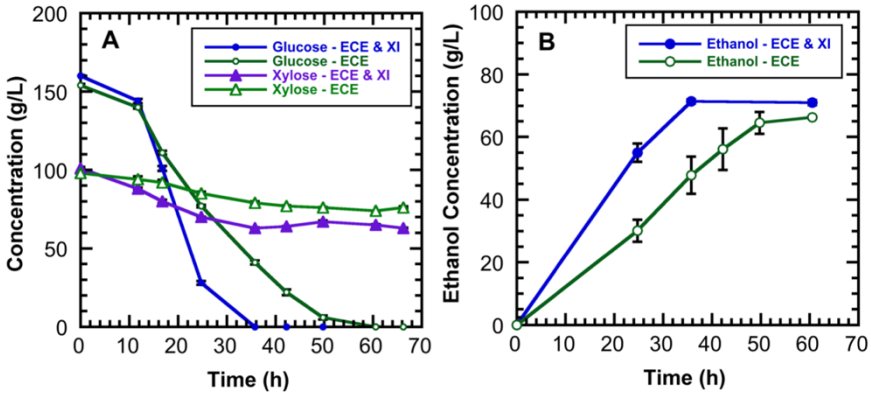

Figure S5: Glucose, xylose, and ethanol concentration profiles for *S. pastorianus* cultured with standard levels of ECE and with and without xylose isomerase. (A) Glucose (●,○) and xylose (▲,△); and **(B)** ethanol (●,○). Both ECE and XI (●, ▲). ECE only (○,△). Error bars represent the standard deviation.

S.4.2 *ECE Addition Effect on Ethanol Productivity*

To determine the impact the ECE addition on ethanol productivity for *S. pastorianus*, cultures were grown on reagent-grade sugars with varying levels of ECE (100%, 50%, 25%, 5%, and 0%) without xylose isomerase. Duplicate culture conditions were examines for the 50%, 25%, and 5% ECE% concentrations; but only single cultures for the 100% and 0% ECE additions. The glucose, xylose, and ethanol profiles are shown in **Figure S6.** The initial sugar concentrations were 150 g/L glucose and 75 g/L xylose. A 1% YPD medium supplement was only added to the 0% ECE addition culture, as otherwise this culture would not have grown [7]. The glucose consumption and ethanol productivity rates were both significantly affected by the ECE amount ($p \leq 0.05$). Interestingly, the 5% ECE addition had the highest glucose consumption rate (4.59 ± 0.10 g glucose/L·h) and the highest ethanol productivity (1.67 ± 0.05 g ethanol /L·h); however, was not considered different from the 25%, or 50% glucose consumption or ethanol production rates. Whereas, the culture without ECE had the lowest glucose consumption rate (3.43 ± 0.10 g glucose/L·h and lowest ethanol production rate (1.21± 0.04 g ethanol /L·h), and was significantly different. As these culture were all conducted without xylose isomerase, it is likely that 5% ECE does not represent the optimum with xylose isomerase present, since all of the rates are lower than observed with both ECE and xylose isomerase present.

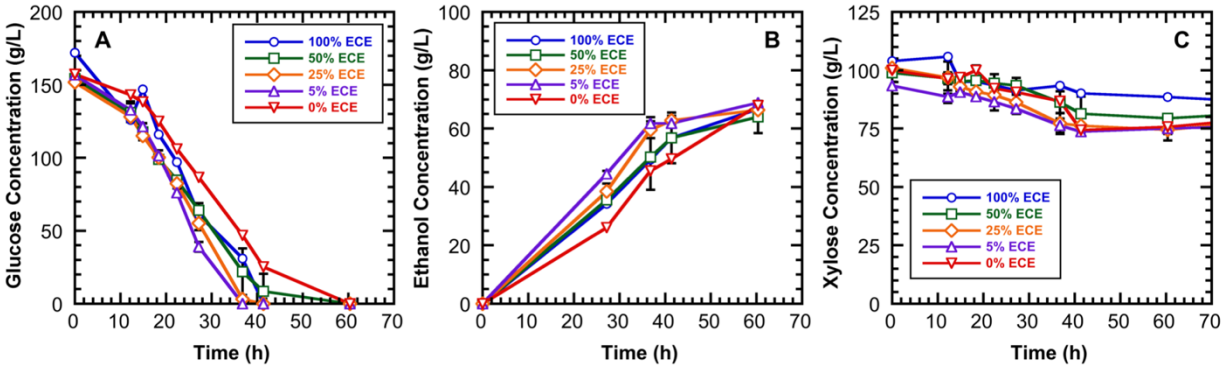

Figure S6: Glucose, ethanol, and xylose concentration profiles for *S. pastorianus* cultured on various amount of ECE without xylose isomerase. (A) Glucose, **(B)** Ethanol, and **(C)** Xylose. ECE: 100% (17 g dcw/L; ○), 50% (□), 25% (◇), 5% (△), and 0% ECE (▽). Singleton cultures were conducted for the 100% and 0% ECE conditions, all other conditions were conducted in triplicate. Error bars represent the standard deviation.

S.4.3 *Xylose Isomerase and ECE Addition Effects on Ethanol Productivity*

The impact xylose isomerase addition on ethanol productivity was quantified for three levels of xylose isomerase (Normal – 5 g/L, Double – 10 g/L, and Half – 2.5 g/L) on reagent-grade sugars with and without ECE. Duplicate cultures were used for the Double and Half xylose isomerase addition, where only single cultures for the Normal xylose isomerase addition. The glucose, xylose, and ethanol profiles are shown in **Figure S7**, where in the initial sugar concentrations were 150 g/L glucose and 75 g/L xylose. The cultures without ECE included a 1% rich media addition to provided micronutrients. The glucose consumption rate and ethanol productivities were both significantly affected by the ECE and the amount of the xylose isomerase ($p \leq 0.05$) and had interaction affects ($p \leq 0.05$). The highest glucose consumption rate (6.47 ± 0.11 g glucose /L·h) and ethanol productivity(2.35 ± 0.03 g ethanol /L·h) was observed for the culture with ECE and Double (10 g/L) xylose isomerase. The glucose consumption rate was significantly higher than the Normal (5 g/L) xylose isomerase culture (5.41 ± 0.04 g glucose/L·h), but was not significantly different for ethanol productivity (2.23 ± 0.03 g ethanol /L·h). This outcome would need to be reevaluated in hydrolysate to determine if economically advantageous to include more xylose isomerase in the fermentation, since xylose isomerase can be reused.

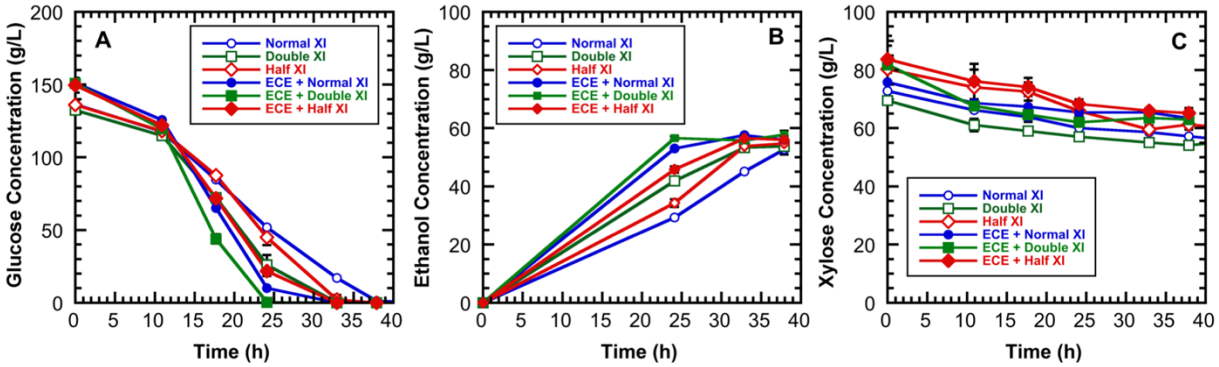

Figure S7: Glucose, ethanol, and xylose concentration profiles for *S. pastorianus* cultured with with and without ECE and varying amounts of xylose isomerase. (A) Glucose, **(B)** Ethanol, and **(C)** Xylose. Without ECE (0 g dcw/L; ○,□,◇); with ECE (17 g dcw/L; ●,■,◆); Xylose isomerase (XI) levels: Normal (5 g/L: ●,○), Double (10 g/L: ■,□), and Half (2.5 g/L: ◆,◇). Singleton cultures were conducted for the Normal XI and ECE + Normal XI, all other conditions were conducted in duplicate. Error bars represent the standard deviation.

Supplemental Material References

1. Gong, C.S.; Chen, L.F.; Flickinger, M.C.; Chiang, L.C.; Tsao, G.T. Production of ethanol from D-xylose by using D-xylose isomerase and yeasts. *Appl. Environ. Microbiol.* **1981**, *41*, 430-436.
2. Neidhardt, F.C.; Umbarger, H.E. Chemical Composition of *Escherichia coli*. In *Escherichia coli and Salmonella*, 2nd ed.; Neidhardt, F.C., Curtiss, R., Lin, E.C.C., Low, K.B., Magasanik, B., Reanikoff, W.S., Riley, M., Schaechter, M., Umbarger, H.E., Eds. ASM Press: Washington D. C., 1996; Vol. 1, pp. 13-16.
3. Bremer, H.; Dennis, P.P. Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate. In *Escherichia coli and Salmonella*, 2nd Edition ed.; Neidhardt, F.C., Curtiss, R., Lin, E.C.C., Low, K.B., Magasanik, B., Reanikoff, W.S., Riley, M., Schaechter, M., Umbarger, H.E., Eds. ASM Press: Washington D. C., 1996; Vol. 2, pp. 1553-1569.
4. Delgado, F.F.; Cermak, N.; Hecht, V.C.; Son, S.; Li, Y.; Knudsen, S.M.; Olcum, S.; Higgins, J.M.; Chen, J.; Grover, W.H., et al. Intracellular Water Exchange for Measuring the Dry Mass, Water

Mass and Changes in Chemical Composition of Living Cells. *Plos One* **2013**, *8*, doi:10.1371/journal.pone.0067590.
5. Taymaz-Nikerel, H.; Borujeni, A.E.; Verheijen, P.J.T.; Heijnen, J.J.; van Gulik, W.M. Genome-Derived Minimal Metabolic Models for *Escherichia coli* MG1655 With Estimated In Vivo Respiratory ATP Stoichiometry. *Biotechnol. Bioeng.* **2010**, *107*, 369-381, doi:10.1002/bit.22802.
6. Gonzalez, J.E.; Long, C.P.; Antoniewicz, M.R. Comprehensive analysis of glucose and xylose metabolism in *Escherichia coli* under aerobic and anaerobic conditions by C-13 metabolic flux analysis. *Metab. Eng.* **2017**, *39*, 9-18, doi:10.1016/j.ymben.2016.11.003.
7. Miller, K.P.; Gowtham, Y.K.; Henson, J.M.; Harcum, S.W. Xylose isomerase improves growth and ethanol production rates from biomass sugars for both *Saccharomyces pastorianus* and *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **2012**, *28*, 669-680, doi:10.1002/btpr.1535.
8. Gowtham, Y.K.; Miller, K.P.; Hodge, D.B.; Henson, J.M.; Harcum, S.W. Novel two-stage fermentation process for bioethanol production using *Saccharomyces pastorianus*. *Biotechnol. Prog.* **2014**, *30*, 300-310, doi:10.1002/btpr.1850.
9. Sharma, S.S.; Campbell, J.W.; Frisch, D.; Blattner, F.R.; Harcum, S.W. Expression of two recombinant chloramphenicol acetyltransferase variants in highly reduced genome *Escherichia coli* strains. *Biotechnol. Bioeng.* **2007**, *98*, 1056-1070.
10. Korz, D.J.; Rinas, U.; Hellmuth, K.; Sanders, E.A.; Deckwer, W.D. Simple fed-batch technique for high cell-density cultivation of *Escherichia coli*. *J. Biotechnol.* **1995**, *39*, 59-65.
11. Xu, B.; Jahic, M.; Blomsten, G.; Enfors, S.O. Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 564-571.
12. Akesson, M.; Karlsson, E.; Hagander, P.; Axelsson, J.; Tocaj, A. On-Line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. *Biotechnol. Bioeng.* **1999**, *64*, 590-598.
13. Jain, A.; Walker, T.H. Pretreatment composition for biomass conversion process. 10.10.2013, 2013.
14. Jain, A.; Wei, Y.; Tietje, A. Biochemical conversion of sugarcane bagasse into bioproducts. *Biomass Bioenergy* **2016**, *93*, 227-242, doi:http://dx.doi.org/10.1016/j.biombioe.2016.07.015.