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High Gravity Fermentation of Sugarcane Bagasse Hydrolysate by *Saccharomyces pastorianus* to Produce Economically Distillable Ethanol Concentrations: Necessity of Medium Components Examined

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Abstract: A major economic obstacle in lignocellulosic ethanol production is the low sugar concentrations in the hydrolysate and subsequent fermentation to economically distillable ethanol concentrations. We have previously demonstrated a two-stage fermentation process that recycles xylose with xylose isomerase to increase ethanol productivity, where the low sugar concentrations in the hydrolysate limit the final ethanol concentrations. In this study, three approaches are combined to increase ethanol concentrations. First, the medium-additive requirements were investigated to reduce ethanol dilution. Second, methods to increase the sugar concentrations in the sugarcane bagasse hydrolysate were undertaken. Third, the two-stage fermentation process was recharacterized with high gravity hydrolysate. It was determined that phosphate and magnesium sulfate are essential to the ethanol fermentation. Additionally, the *Escherichia coli* extract and xylose isomerase additions were shown to significantly increase ethanol productivity. Finally, the fermentation on hydrolysate had only slightly lower productivity than the reagent-grade sugar fermentation; however, both fermentations had similar final ethanol concentrations. The present work demonstrates the capability to produce ethanol from high gravity sugarcane bagasse hydrolysate using *Saccharomyces pastorianus* with low yeast inoculum in minimal medium. Moreover, ethanol productivities were on par with pilot-scale commercial starch-based facilities, even when the yeast biomass production stage was included.

Keywords: sugarcane bagasse; *Escherichia coli*; ethanol; xylose isomerase; yeast

1. Introduction

The two major economic obstacles for ethanol production from lignocellulosic feedstock are the conversion of cellulose and hemicellulose into soluble sugars, glucose and xylose, and the subsequent conversion of xylose to ethanol [1–4]. Glucose is readily fermented to ethanol by the *Saccharomyces* species, but most native *Saccharomyces* species cannot metabolize xylose directly to ethanol [5]. Several non-*Saccharomyces* yeast species can metabolize xylose to ethanol, except the conversion rates are very slow compared to *Saccharomyces* glucose-ethanol fermentation rates [6]. Moreover, most acidic- and alkaline-based pretreatment processes yield lignocellulosic hydrolysates that contain high amounts of furan and other phenolic compounds, which are often inhibitory for ethanol production by *Saccharomyces cerevisiae* [7]. In contrast, *S. pastorianus* has demonstrated a higher tolerance to hydrolysate inhibitory components [8,9] and wider growth temperatures than reported for *S. cerevisiae* [10–12]. Furthermore, the glucose and fructose transporters in *S. pastorianus* appear to be unique [13] and do not exhibit diauxic behavior [14]. This allows for glucose and fructose

to be simultaneous used, as xylose isomerase can convert glucose to fructose (acting as a glucose isomerase), even if only slowly at the fermentation temperature and pH [14] since the optimum pH and temperature for xylose isomerase activity is pH 6 to 8 and 70 °C, respectively. This conversion of glucose to fructose enables both sugars to enter glycolysis simultaneously and generate higher amounts of NADH, forcing the cells to recycle the NADH to NAD^+ at a higher rate, and generate ethanol at higher rates [14].

Economical production of bioethanol requires the final ethanol concentration to be at least 70 g/L [2,15,16]. Moreover, the economics improve if xylose can be utilized [16]. To reach these ethanol levels, the sugar concentrations in the hydrolysate must be at least 140 g/L, if one assumes near theoretical conversion of the sugars to ethanol [17]; however, most groups report approximately 80% of theoretical conversion for hydrolysate-derived and starch-derived glucose [16,18]. Additionally, the ethanol fermentation times need to be significantly less than 80 h for ethanol productivity to be considered economically viable [2,15]. In some cases, to decrease the fermentation time, very high yeast loadings (or inocula) have been used (50- to 100-g dry cell weight) [19,20]; however, the costs associated with these high yeast loadings, in terms of the glucose used to generate these cells, has not always been accounted for in the ethanol yields. These differences in inoculum amounts can make a direct comparison of overall yields difficult between studies.

Alternatively, recombinant approaches have been used to genetically modify *S. cerevisiae* and *S. pastorianus* to be able to metabolize xylose directly. Many of these xylose metabolism modifications studies have not yet demonstrated higher ethanol productivity or ethanol concentrations, in part, due to using sugar concentrations that are too low in the fermentations [21,22]. Also, there have been recent gains in improving *S. cerevisiae* ethanol and inhibitor tolerance via recombinant means [23–29]. Complementary approaches have coupled cellobiose utilization with xylose utilization in recombinant *S. cerevisiae* with improved xylose to ethanol conversion rates [30]. Recombinant approaches have not yet yielded a successful approach to xylose utilization that overall surpasses the ethanol productivities of native species on glucose; however, work is getting closer [8,14,31–36].

The focus of this work is to demonstrate the potential economic feasibility of the two-stage fermentation process under high gravity conditions using *S. pastorianus* to achieve high ethanol productivities and high ethanol concentrations on sugarcane bagasse hydrolysate as the sugar source. The effects of medium component additives on ethanol productivity and yields were examined, as well as the requirements for the *E. coli* extract (ECE) and xylose isomerase supplements on ethanol productivity under the high gravity fermentation conditions. ECE is heat-killed *E. coli* biomass that is cultured on xylose in fed-batch fermentations. Reagent-grade sugars were used as the substrate in parallel fermentations to benchmark the process for the high gravity sugarcane bagasse hydrolysate fermentations.

2. Materials and Methods

2.1. Microorganisms

Saccharomyces pastorianus was obtained from Winemakeri, Inc. (Beaverbank, NS, Canada) and is commercially sold as Liquor Quik Super Yeast X-Press. *Escherichia coli* strain MG1655 (ATCC 700296) was obtained from American Type Culture Collection. All stock cultures were stored at −80 °C until cultured.

2.2. Medium Components

The minimal medium was modified from [37] and described in [38]. The minimal medium contained a buffer solution (80 g/L KH_2PO_4, 40 g/L $(NH_4)_2HPO_4$), and 17 g/L citric acid), which was modified to be a 20 × stock in this work, a 500 × magnesium sulfate (200 g/L $MgSO_4 \cdot 7\,H_2O$) solution, a 100 × trace metal solution (1.5 g/L $MnCl_2 \cdot 4\,H_2O$, 1.3 g/L $Zn(CH_3COO)_2 \cdot 2\,H_2O$, 0.3 g/L H_3BO_3, 0.24 g/L $Na_2MoO_4 \cdot 2\,H_2O$, 0.25 g/L $CoCl_2 \cdot 6\,H_2O$, 0.15 g/L $CuCl_2 \cdot 2\,H_2O$, and 0.84 g/L EDTA), and a 100 × iron

(III) citrate (1 g/L) solution, where the final medium was pH 5.8. The carbon sources were glucose, xylose, or hydrolysate, as specified. When *S. pastorianus* cultures were fermented on reagent-grade sugars, a rich-medium addition was required in the minimal medium to provide micronutrients. This rich-medium addition (final concentration of 1% YPD medium by volume) was adapted from the yeast-peptone-dextrose (YPD) medium, as described in Gong et al. (1981) and contained 10 g/L yeast extract and 20 g/L peptone without glucose [39]. The chemicals KH_2PO_4, $(NH_4)_2HPO_4$, $MnCl_2 \cdot 4\,H_2O$, $Zn(CH_3COO)_2 \cdot 2\,H_2O$, H_3BO_3, glucose, and citric acid were purchased from ThermoFisher; magnesium sulfate, $CoCl_2 \cdot 6\,H_2O$, iron (III) citrate, and peptone were purchased from Sigma; yeast extract, xylose, $Na_2MoO_4 \cdot 2\,H_2O$, EDTA, and $CuCl_2 \cdot 2\,H_2O$ were purchased from Acros. Xylose isomerase was obtained from Sigma (G4116). All chemicals were ACS certified or ≥99% pure, except the yeast extract and peptone, which were microbiological certified.

2.3. E. coli Extract (ECE)

The *E. coli* cell extract (ECE) production process has been described previously [8]. Briefly, *E. coli* were grown aerobically a 5-L BioStatB fermenter (Sartorius) at 37 °C in minimal medium containing xylose. An exponential feed profile was used to control the growth rate by maintaining a xylose limited culture [38,40,41]. At the end of the fermentation, the *E. coli* biomass was killed by heating to 65 °C for 60 min. The heat-killed *E. coli* cell extract biomass slurries were stored at −20 °C and are referred to as ECE [8,14]. The *E. coli* fermentations on xylose as the sole carbon source were further characterized and optimized and this information can be found in the Supplemental Materials, Section S1. Additionally, there are several literature reports that describe the chemical composition and components found in *E. coli* cells [42–46].

2.4. Sugarcane Bagasse and the Pre-Treatment Process

Sugarcane bagasse was obtained from the Audubon Sugar Institute at Louisiana State University, St. Gabriel, LA, USA (donated by Dr. Donal F. Day). Table 1 lists the compositional analysis of the sugarcane bagasse [47]. The sugarcane bagasse was pre-treated by the ethanol-potassium hydroxide-hydrogen peroxide (EHO) method and enzymatically hydrolyzed as described in the patent application [48] and Jain et al. (2016) [47]. Briefly, the pretreatment used 200 g bagasse (dry basis, 606 g total, which contained 406 g moisture), 1400 mL of 95% ethanol, 144 mL 35% hydrogen peroxide, and 50 g potassium hydroxide. The bagasse was manually mixed prior to sealing the vessel. The reaction incubated for 1-h at approximately 77 °C in a Parr floor-stand pressure reactor system (Model 4553) with a Parr controller (Model 4843). Mixing was maintained at approximately a revolution per second during the reaction. Four consecutive cold tap water washes were used (7-L each). The pre-treated bagasse was pressed dry to a final moisture content of approximately 76%. The pre-treated bagasse was frozen at −20 °C. Table 1 lists the composition of pretreated bagasse.

Table 1. Composition of sugarcane bagasse and pretreated bagasse. On a dry basis, the untreated bagasse (281.9 ± 0.12 g dry weight) resulted in 160.66 ± 4.50 g dry weight pretreated bagasse (adapted from Jain et al. (2016) [47]).

Component	Composition (Weight %)	
	Untreated Bagasse	**Pretreated Bagasse**
Glucan	41.34 ± 0.49	66.23 ± 0.33
Xylan	13.21 ± 0.17	17.19 ± 0.19
Arabinan	1.61 ± 0.14	2.34 ± 0.13
Lignin	20.66 ± 0.5	6.06 ± 0.17
Ash	6.76 ± 0.47	6.77 ± 0.48
Extractive	11.12 ± 0.34	—
Solid residues (g)	94.70 ± 2.12	98.58 ± 1.30

2.5. Enzyme Hydrolysis Conditions

The standard hydrolysate was prepared as described in Jain and Walker [47]. The hydrolysate with 123 g/L glucose and 44 g/L xylose was prepared using the "improved batch" process that reduced water additions to the reaction mixture. See Supplemental Materials Section S2 for more details. The high gravity hydrolysates (glucose concentration greater than 160 g/L) were prepared using a fed-batch reaction process with an initial addition of 90 g (total mass) pre-treated bagasse (68% or 70% moisture depending on the batch). Each batch contained 12 mL "low activity" CTec2 and 9.5 mL citrate buffer and was incubated at 50 °C, pH 4.8, and 200 rpm. At 24 and 48 h, an additional 90 g of pre-treated bagasse was added for a total of 270 g bagasse per batch. The entire batch was incubated for 72 h.

2.6. S. Pastorianus Fermentations

Overnight cultures of *S. pastorianus* grown aerobically on glucose minimal medium were used to inoculate the shake flasks fermentations on reagent-grade sugars (glucose and xylose) or the sugarcane bagasse hydrolysate (also containing both glucose and xylose). Fermentations were performed at 30 °C in 125 mL shake-flasks with 25 mL working volumes sealed with rubber stoppers that contained a one-way air valve and a sampling syringe, which maintained the anaerobic environment and allowed the carbon dioxide to exit the shake-flask. Cultures were grown in an orbital shaker and agitated at 130 rpm. The initial cell densities in the fermentations were approximately 0.5 OD, corresponding to a dry cell weight (dcw) of 0.25 g dcw/L.

Prior to fermentations, the hydrolysate pH was adjusted to 5.65 with 10 M sodium hydroxide. The hydrolysate replaced the water and sugars in the minimal medium. The basic medium formulation for a 25-mL fermentation is given in Table 2, as also described in Miller et al. (2012) and Gowtham et al. (2014) [8,14]. Reagent-grade sugar control fermentations were run in parallel, where the hydrolysate was replaced by water, glucose, xylose, and 0.25 mL YPD medium. When required, water was used to normalize the initial sugar concentrations between cultures with different media additions.

Table 2. Component additions for a 25-mL shake flask fermentation. The water, glucose, and xylose solution volumes required for a reagent-grade sugar fermentation depend on the sugar concentrations in the hydrolysate. For illustration, the target concentrations for the reagent fermentation broth were 186 g/L glucose and 51 g/L xylose.

Component	Amount in Shake Flask (25 mL)	
	Hydrolysate	Reagent-Grade Sugar
Hydrolysate (sugar concentration variable)	21.7 mL	-
Water	-	9.60 mL
Glucose solution (500 g/L)	-	9.30 mL
Xylose solution (500 g/L)	-	2.55 mL
Phosphate buffer, pH 5.8 (20×)	1.25 mL	1.25 mL
Trace metals solution (100×)	0.25 mL	0.25 mL
Iron(III) citrate solution (100×)	0.25 mL	0.25 mL
Magnesium sulfate solution (500×)	0.05 mL	0.05 mL
ECE (17 g dcw/L)	1.50 mL	1.50 mL
Xylose isomerase	0.125 g	0.125 g
YPD medium	-	0.25 mL

2.7. Analytical Methods

Cell concentrations were measured as cell densities (OD) by absorbance at 600 nm with a Spectronic 20 Genesys instrument (Spectronic Instruments, UK). Sugars and ethanol samples were taken periodically for analysis throughout the fermentations (1.5 mL samples). These samples were centrifuged to separate the pellet and supernatant. The supernatant was collected and frozen for later analysis. Glucose and xylose concentrations were determined using a Yellow Springs

Instruments 2900 biochemistry analyzer [8]. An Agilent 7890A GC system was used to measure ethanol concentrations [14]. The supernatant and calibration standards were filtered through a 0.2 μm syringe filter prior to analysis.

2.8. Statistical Analyses

Statistical analysis was performed using the software JMP Pro 10 (SAS Institute Inc., Cary, NC), where replicate cultures or reactions were conducted. Data were analyzed using the GLM procedure ($p \leq 0.05$) and least squares mean differences with Student's *t*-tests. Statistical analysis of responses included glucose consumption rates, final ethanol concentrations, ethanol yields, xylose conversions, and ethanol productivities. The effectors were media additives, ECE, and xylose isomerase. Standard errors are reported initially for concentrations, consumption rates, yields, and productivities. Error bars on graphs represent standard deviations, as it was determined that error bars that represented standard error were too small to see in many cases.

3. Results

Our previous work has demonstrated that *S. pastorianus* can grow under high gravity fermentation conditions and produce high concentrations of ethanol on reagent-grade sugars (>150 g/L) [14,49] as part of a two-stage fermentation process. An updated schematic of the two-stage fermentation process is included in the Supplemental Materials (Figure S3). Additionally, *S. pastorianus* are able to consume both fructose and glucose at enhanced rates when xylose isomerase is present in the medium, which resulted in the near theoretical conversion of glucose to ethanol [8,14]. Further, the recycling of the xylose was demonstrated as ECE increased the glucose consumption and ethanol productivity for low sugar concentration corn stover hydrolysate by *S. pastorianus* [8]. However, due to low glucose concentration in the corn stover hydrolysate (less than 30 g/L glucose), high gravity fermentation conditions were not evaluated at that time, nor the potential inhibitor effects. The Supplemental Materials describe the screen experiments used to develop the fed-batch hydrolysis reaction to generate hydrolysate with high gravity sugar concentrations (Figure S2, Section S2). As these screen experiments were conducted in parallel with the medium component analysis, the glucose and xylose concentrations in the hydrolysate are clearly indicated.

3.1. Medium Component Effects on Ethanol Productivity

The minimal medium used in the yeast fermentations was originally developed for *E. coli* high cell density fermentations, and likely contains components that are not necessary for yeast cultured on hydrolysates [8,14,37]. Consequently, the first part of this study was to determine which minimal medium components were necessary for ethanol production using sugarcane bagasse hydrolysates that were pre-treated using the EHO method. One of the goals of this component analysis is to reduce the dilution of the fermentation medium by the water that accompanies these components, as all of the media components are prepared separately and used from stock solutions.

Sources of water in the original minimal medium recipe comes from the four major component stock solutions that are prepared separately due to issues of precipitation if autoclaved together [37]. Table 2 lists these four major component stock solutions and volumes added per shake flask culture. For the yeast ethanol fermentations, the hydrolysate and ECE replace the water and sugars in the standard medium formula. Therefore, reducing or eliminating one of these four medium components would allow for more hydrolysate to be added to the medium, and thus potentially increase the final ethanol concentration.

To assess the elimination of these dilution sources, *S. pastorianus* were cultured in media formulated using sugarcane bagasse hydrolysate containing 65 g/L glucose and 20 g/L xylose. The yeast-peptone-dextrose (YPD) medium additions were not included, as the hydrolysate and ECE contain sufficient micronutrients to support yeast growth and ethanol production. The glucose and ethanol profiles for the medium component effect experiments are shown in Figure 1. The initial

glucose concentration in the fermentation broth was 51.5 ± 0.02 g/L. By calculation, the xylose concentration was about 16 g/L. The matrix effect for the xylose measurement due to the ECE resulted in non-meaningful values, which for this study, xylose quantification was determined to be non-critical. The glucose consumption rate was fastest in the complete medium, where the culture with only the Mg solution addition was the second-fastest. The ethanol concentration profiles also showed the highest productivity for the complete medium, where the culture with the Mg solution had only a slightly lower ethanol productivity. Interestingly, the iron and trace solution addition cultures proceeded no better than the culture without any additions (None) with respect to glucose consumption. Yet these three cultures (None, trace, and iron) generated similar levels of ethanol as the other cultures, although much slower, indicating *S. pastorianus* have the capability to generate ethanol without additives from hydrolysate when ECE and xylose isomerase are supplemented.
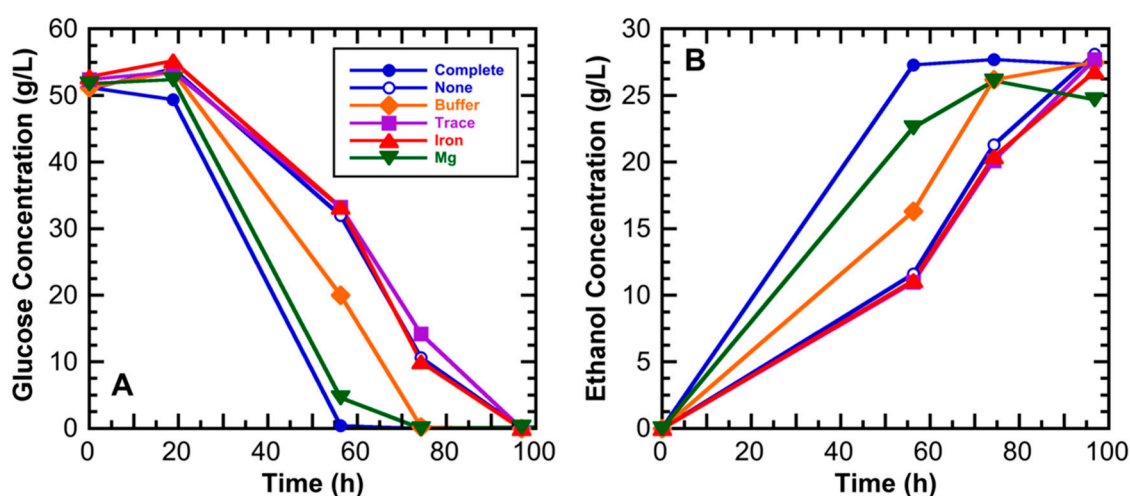
Figure 1. Effect of medium components on the glucose consumption rates and ethanol productivities for *S. pastorianus* on sugarcane bagasse hydrolysate. (**A**) Glucose; (**B**) ethanol. *S. pastorianus* was cultured on sugarcane bagasse hydrolysate that initially contained 65 g/L glucose. Cultures in standard medium (Complete, ●), none of the medium components (None, ○), phosphate buffer only (Buffer, ♦), trace metal solution only (Trace, ■), iron citrate solution only (Iron, ▲), and magnesium sulfate solution only (Mg, ▼). The legend for both panels is shown in (**A**). Only single cultures were conducted for each condition as this was a screening study.

Since the Mg solution addition culture performed well and was only slightly slower than the complete medium culture, the effect of the phosphate buffer solution was investigated. A second set of fermentations was conducted where the phosphate buffer concentration was varied while holding the Mg concentration constant. The glucose profiles are shown in Figure 2 for the phosphate buffer ranging experiment. In this case, the initial glucose concentration in the fermentation broth was 45 g/L glucose, as this was a different batch of sugarcane hydrolysate. The glucose consumption rate was highest in the complete medium, where the Mg solution only culture had the lowest glucose consumption rate. The culture with the Mg solution plus 1× phosphate buffer had glucose consumption rates equal to the culture in the complete medium. Likewise, the ethanol production rates were observed to be the same for the complete medium and Mg solution plus 1× Buffer cultures, whereas the Mg solution only and the Mg solution plus 0.1× Buffer were significantly lower. Interestingly, the xylose profiles for the four fermentations are not significantly different. The matrix effect caused by the hydrolysate on the xylose measurement makes precise xylose concentration measurement difficult; however, the trends are clear, and the xylose was consumed only slightly during the fermentation, as expected, due to minimal xylose isomerase activity at the fermentation temperature and pH conditions. Thus, the Mg solution plus 1× Buffer was determined to be sufficient for high glucose consumption and ethanol productivities on sugarcane bagasse hydrolysate. Since the hydrolysate sugar concentrations used

in these medium addition studies are too low to support fermentations that would reach over 70 g/L ethanol (high gravity), parallel work was conducted in-house to increase the sugar concentrations in the hydrolysate (details in the Supplemental Materials, Section S2).
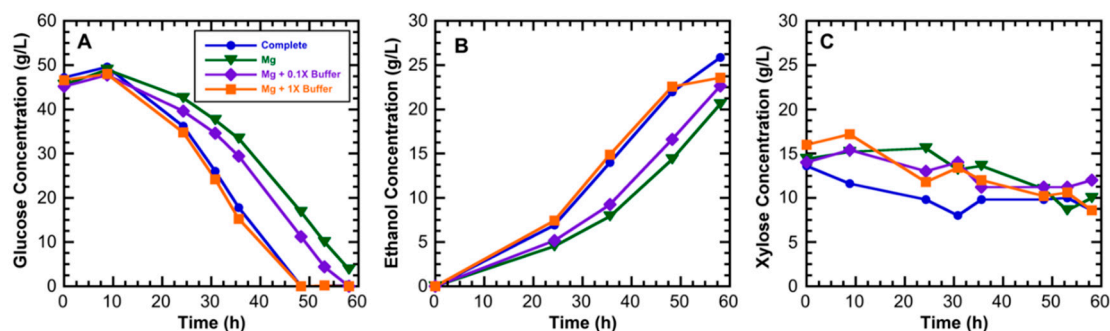

Figure 2. Effect of the phosphate buffer on the glucose consumption rates and ethanol productivities for *S. pastorianus* on sugarcane bagasse hydrolysate. (**A**) Glucose; (**B**) ethanol; (**C**) xylose. Cultures in standard medium (Complete, ●); magnesium sulfate solution (Mg, ▼); magnesium sulfate solution and one-tenth the buffer concentration (Mg + 0.1× Buffer, ♦); and magnesium sulfate solution and the normal buffer concentration (Mg + 1× Buffer, ■). The legend for all panels is shown in (**A**). Only single cultures were conducted for each condition as this was a screening study.

As shown in Table 2, the addition of ECE to the fermentation medium has a significant dilution effect. Thus, increasing the biomass concentration of the *E. coli* in the ECE would reduce the volume of ECE required (work described in the Supplemental Materials, Section S1). In parallel, the necessity for ECE in the more concentrated hydrolysate was assess for the on the hydrolysate containing 65 g/L glucose and 20 g/L xylose. This resulted in a fermentation broth with only 50 g/L glucose. Figure 3 shows the glucose and ethanol profiles for *S. pastorianus* on sugarcane bagasse hydroxylate with and without the ECE addition. Xylose isomerase was included as it is not considered to be a nutrient source. Despite the significant dilution of the sugars by the ECE addition, the ethanol productivity was significantly better for the culture with ECE and xylose isomerase additions compared to the cultures without ECE (fermentation times less than 48 h versus 300 h). As has been previously observed in the low sugar and reagent-grade sugar fermentations [8,14], the final ethanol yields are approximately equal. Moreover, ethanol fermentation optimization studies for the amount of ECE and xylose isomerase, conducted in reagent-grade sugars, can be found in the Supplemental Materials (Section S4). These data together indicate that significant optimization of the fermentation process with ECE and xylose isomerase is still possible.

3.2. High Gravity Fermentations Using Sugarcane Bagasse Hydrolysate

The first high gravity hydrolysate was prepared using an improved hydrolysis batch reaction process. This hydrolysate contained 123 g/L glucose and 44 g/L xylose, which resulted in fermentation media of approximately 94 g/L glucose and 31 g/L xylose. Due to the limited size of the batch hydrolysate reaction (<17 mL), the hydrolysate culture was conducted as a singleton, as a single shake flask culture can hold up to 21.7 mL hydrolysate (see the Supplemental Materials for the reaction conditions; Table S3). As this hydrolysate had significantly higher sugar concentrations than had been previously obtained, the effects of inhibitors was the main concern. Single cultures of each condition were conducted in parallel using the hydrolysate and reagent-grade sugars. Figure 4 shows the glucose, xylose, and ethanol profiles for the "improved" hydrolysate. The glucose and ethanol profiles are similar for the hydrolysate and reagent-grade sugar fermentations, which indicates that inhibitory effects were minimal even at these higher sugar concentrations. The xylose conversion was higher in the reagent-grade sugar fermentations, which has been previously observed [8]. However, the glucose

and xylose concentrations were insufficient to achieve 70 g/L ethanol, even if theoretical yields were realized [50].
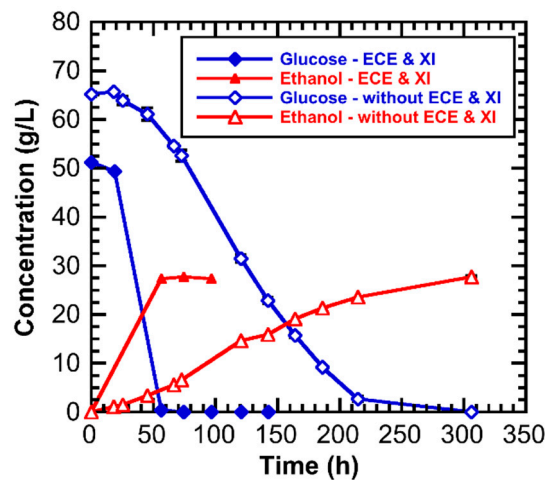

Figure 3. Effect of *E. coli* extract (ECE) and xylose isomerase on the glucose consumption rate and ethanol productivity for *S. pastorianus* cultured on sugarcane bagasse hydrolysate. Glucose (♦,◇); ethanol (▲,△). With ECE and xylose isomerase (♦,▲); without ECE or xylose isomerase (XI) (◇,△). The cultures were conducted in triplicate; however, ethanol was only evaluated for every third sample, except for the last sample, and for those cultures, all three samples were evaluated. Error bars represent the standard deviation.
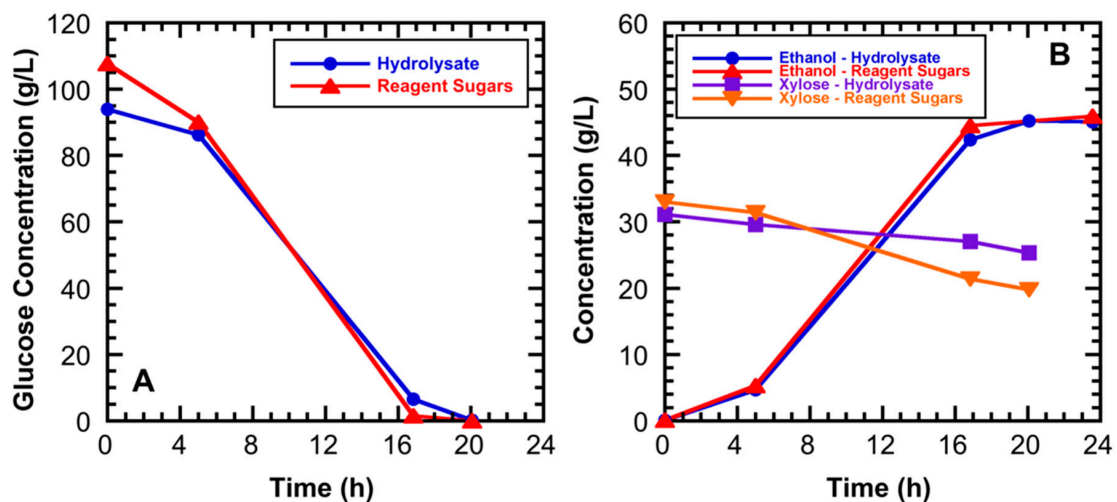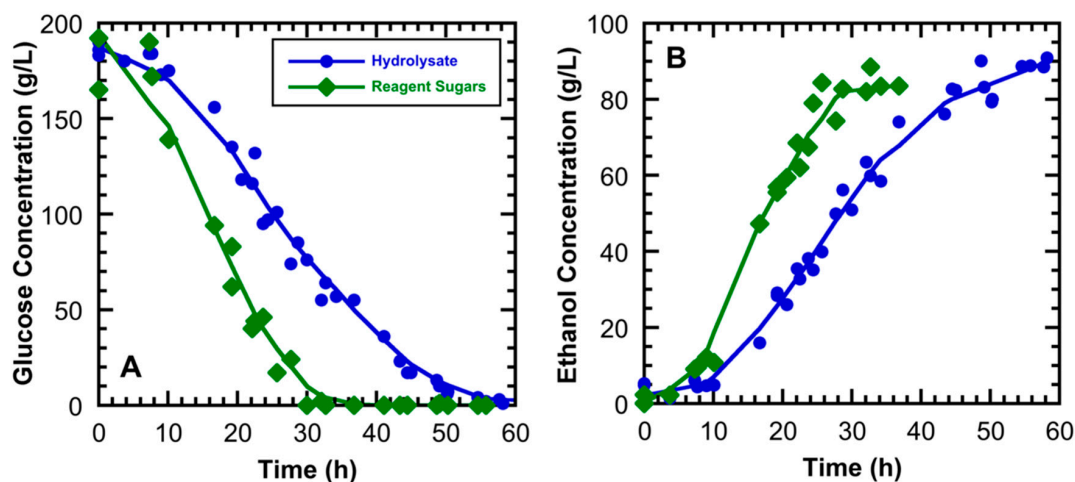

Figure 4. Comparison of fermentation efficiency and kinetics for sugarcane bagasse hydrolysate and reagent-grade sugar fermentations by *S. pastorianus*. (**A**) Glucose (●,▲); (**B**) ethanol (●,▲) and xylose (■,▼). The hydrolysate concentration was 123 g/L glucose and 44 g/L xylose, which resulted in fermentation media of approximately 94 g/L glucose and 31 g/L xylose. Cultures in sugarcane bagasse hydrolysate (Hydrolysate; ●,■). Cultures in reagent-grade sugars (Reagent Sugars; ▲,▼). These two conditions were each conducted as singletons.

Using the hydrolysate prepared by the fed-batch hydrolysis reaction process, four replicate fermentations for hydrolysate and reagent-grade sugars were conducted using a common inocula for each pair. The sugar concentrations in the hydrolysate was 201 and 55 g/L xylose (the Supplemental Materials describe the steps used to develop this process in greater detail). The hydrolysate sugar concentrations in this material have the potential to generate over 70 g/L ethanol in fermentation [50]. The initial glucose concentrations in the fermentation broths were 186 ± 1.3 and 183 ± 12 g/L for the hydrolysate and reagent-grade sugars, respectively. Figure 5 shows the glucose and ethanol

profiles for these fermentations. The final ethanol concentrations were 89.2 ± 0.5 and 84.3 ± 1.4 g/L from the hydrolysate and reagent-grade sugars, respectively. The ethanol yields calculated based on glucose were approximately 48% and 46% from the hydrolysate and reagent-grade sugar, respectively. These yields includes the glucose used to generate the yeast biomass as the inocula were 0.25 g dcw/L. The ethanol productivity from the hydrolysate was 1.62 ± 0.01 g/L·h, which also includes the time required to grow the cells.

Figure 5. Comparison of fermentation efficiency and kinetics on high gravity hydrolysate and reagent-grade sugar fermentations by *S. pastorianus*. (**A**) Glucose; (**B**) ethanol. Cultures in sugarcane bagasse hydrolysate (Hydrolysate, ●). Cultures in reagent-grade sugars (Reagent Sugars, ♦). Data represent the combined data from four replicate cultures per culture condition; all data points shown. To obtain a uniform hydrolysate, the hydrolysates from four fed-batch reactions were combined. The reagent-grade sugar fermentations were run, pairwise in parallel, with the same inoculum. The legend for both panels is shown in panel (**A**). All data points are shown for the four replicates. The glucose consumption rates and ethanol productivities are significantly different ($p \leq$ 0.05); however, the ethanol yields are not significantly different ($p > 0.05$).

3.3. Effect of ECE and Xylose Isomerase on the Hydrolysate Fermentation Kinetics

The need for the ECE and xylose isomerase supplements was re-examined for the high gravity sugarcane bagasse hydrolysate fermentations. In particular, the goal of this set of fermentations was to confirm that the ECE and xylose isomerase additions still increased the glucose consumption rate and the ethanol productivity under high gravity fermentation conditions. Previous studies on reagent-grade sugars have demonstrated that both the ECE and xylose isomerase have significant effects on ethanol productivity in high gravity fermentations (Supplemental Materials, Section S4), but this effect has not be demonstrated on high gravity hydroxylates. The enzyme-treated hydrolysate used for this comparison was generated by the fed-batch process; however, from a different batch than used in Figure 5. The hydrolysate used to examine the effects of the ECE and xylose isomerase had sugar concentrations of 162 g/L glucose and 45 g/L xylose, which resulted in a fermentation media with 148.2 ± 0.14 g/L glucose. Since the goal of these fermentations was only to demonstrate if a significance difference existed due to the ECE and xylose isomerase for the glucose consumption rate and ethanol productivity, the sample analysis was streamlined to two times. The time profiles from Figures 4 and 5 were used to selected these two time points, with the objective of capturing the fermentation prior to completion. The sample times selected accounted for the lower initial sugar concentrations (148 vs. 186 g/L), and the times 36 and 45 h were selected. Figure 6 shows the glucose and ethanol profiles for these cultures, and clearly demonstrate that the ECE and xylose isomerase supplements significantly increased the glucose consumption rate and ethanol productivity ($p \leq 0.05$). The ethanol concentration reached 82.4 ± 0.33 g/L ethanol in less than 45 h when ECE and xylose isomerase were present versus

59.3 ± 0.02 g/L for the cultures without additions.The ethanol productivity for the cultures with ECE and xylose isomerase were 1.89 ± 0.01 g/L·h with a yield of 56.2% ± 0.3% from glucose, which includes the time required to grow the cells from the 0.05 g dcw/L inoculum. In comparison, the cultures without the additives have ethanol yields from glucose of 51.2% ± 0.04% and ethanol productivities of 1.29 ± 0.001 g/L·h. The greatest contribution the ECE and xylose isomerase provides is towards improved ethanol productivity.
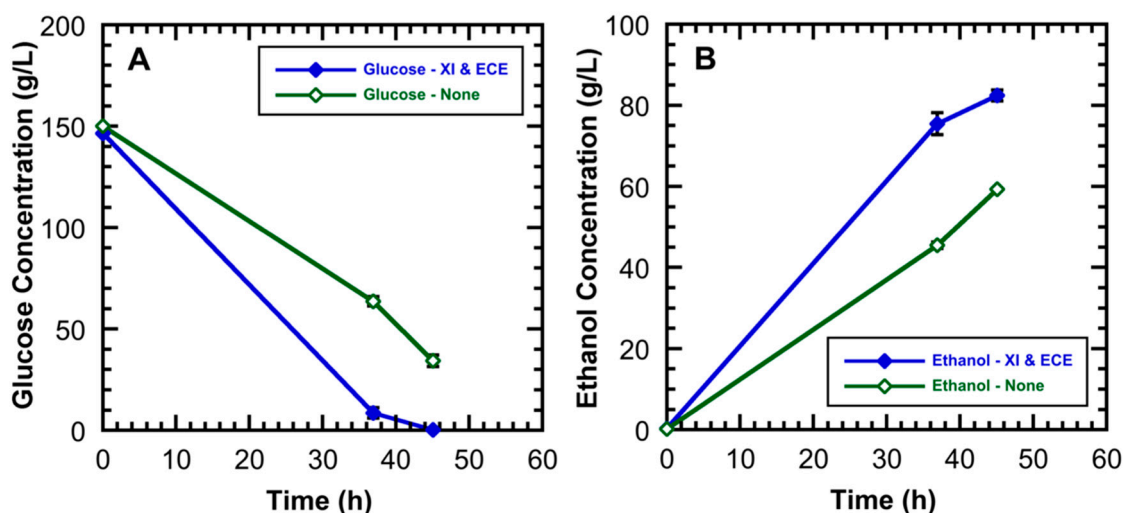
Figure 6. Effect of ECE and xylose isomerase on glucose consumption and ethanol productivity by *S. pastorianus*. (**A**) Glucose; (**B**) ethanol. *S. pastorianus* were cultured on hydrolysate with and without the ECE and xylose isomerase to determine if the species was significant to high gravity hydrolysate fermentation outcomes. Both ECE and xylose isomerase (ECE & XI, ♦); No ECE or xylose isomerase (None, ◇). Duplicate cultures for both conditions were conducted. Error bars represent the standard deviation, which have been included on all data points, but are difficult to see in many cases due to the relative size of the bars to symbols.

4. Discussion

As we have demonstrated previously, on reagent-grade sugars, the ECE and xylose isomerase significantly increase glucose consumption rates and ethanol productivities [8,14]. As expected, the reagent-grade sugar fermentations consumed the glucose and produced ethanol within about 35 h (Figure 5), while the hydrolysate fermentations took approximately 55 h to completely consume the glucose and reach maximum ethanol levels. These data indicate that the inhibitor effects were minimal and *S. pastorianus* in the system was robust; whereas for native *S. cerevisiae,* tolerance is a sufficient concern that genomic approaches have been taken to try to solve the lack of inhibitor tolerance issue [23,27–29,51]. Another positive attribute of this process is that the required inoculum was only 0.25 g dcw/L (0.5 OD), which represents a very low inoculum within the biofuels community [29,52]. Further, the ethanol productivity reported in this study includes the growth phase as well as the production phase without the addition of yeast extract or peptone. Moreover, this process was competitive with starch-based processes [53]. Further, several recombinant yeast studies that have reported using high density (20 OD) inoculum [25,26] did not include in the ethanol yields or productivities the material and time required to generate the inoculum. In many other ethanol-from-lignocellulosic studies, it is common to use high inoculum, supplementing the medium with yeast extract and peptone [29,32,51,52], corn steep liquor [35], or other nutrients [54]. It is not always clear if the inocula or these added carbon/nutrient sources are included in the total carbon balances when yields are reported. The final ethanol concentrations and the yields reached in this work using ECE and xylose isomerase represent extremely competitive values, surpassing any values reported for recombinant yeast cultures on hydrolysates, evolutionarily adapted, and combined saccharification/fermentation systems [7,23,26,28,30,31,51,54–56].

It was surprising, in this work, that ethanol yield calculated based solely on glucose exceeds the theoretical yield of 51% [57]; however, it is very likely other carbon sources are being used for ethanol production derived from the hydrolysate or the ECE. Even though the final ethanol yields for the cultures with and without ECE and xylose isomerase at the lower hydrolysate concentrations were not different (Figure 3), it is possible that differences in ethanol productivities could be observed in a high gravity fermentations. For example, in this study, in the fermentation without ECE and xylose isomerase on the high-gravity hydrolysate (Figure 6), the yield on glucose to ethanol was significant lower. Had these culture gone to completion, it might have been possible to detect the ethanol final yield differences. Moreover, if the xylose accounted for in the hydrolysate is included in the yield calculation, the conversion of total sugars (190 g/L) was converted to 82 g/L ethanol, which is a yield of 43% or approximately 85% of theoretical yield. In comparison to pilot-scale commercial rice starch fermentations, which use very high gravity fermentation conditions, the high gravity sugarcane bagasse hydrolysate fermentations reported here had comparable ethanol productivities (between 1.6 and 1.8 g/L·h). Additionally, the yields reported for rice starch fermentations are around 83% [18]. It is not unexpected that reported final ethanol concentrations for rice starch fermentations are higher since these are very high gravity fermentations, and not limited by the available sugars in the solution. Thus, the focus of future hydrolysate ethanol fermentation work should emphasis increasing the sugar concentrations in the hydrolysate.

In this work, native *S. pastorianus* has demonstrated the capability to produce ethanol from sugarcane bagasse hydrolysate under high gravity fermentation conditions. The specifics of this processes allow for very low inoculums to be used, corresponding to 0.25 g dcw/L. This has several implications on the economics. First, the yeast biomass production was accounted for in the conversion of glucose to ethanol. Conversely, most reported ethanol fermentations use yeast inoculums on the order of 10 to 50 g dcw/L (or 1.75 to 3.5×10^7 cell/mL) [18,30,32,34–36,58,59]. In these high inoculum cases, it is not apparent that the yield from sugar to ethanol accounts for the sugar used to generate the biomass, which for the 10 g/L case would be at least 20 g/L of glucose. Second, the capability to generate the yeast cells in-fermenter within the normal fermentation timeframe eliminates the need to purchase yeast, which a cost-savings, and allows for process to be located remotely and not rely on yeast biomass delivery. Third, this process did not include the common media additions of yeast extract or peptone [21,22,31,52]. This further reduces the reliance of the process on outside sources for materials and closes the carbon balance better than when these nutrient sources are added to the fermentation but not accounted for in the carbon balance. Ultimately, recycling the xylose back to the yeast as a nutrient-rich supplement allows for a full accounting of the sugars and other carbon sources that enter the system to be directly compared to the ethanol generated. Finally, the process described here represents an economically competitive process that could still be improved by increasing the sugar content in the hydrolysate and examining the ECE and xylose isomerase levels required in the hydrolysate fermentation for optimal performance.

5. Conclusions

Native *S. pastorianus* have the capability to produce ethanol from sugarcane bagasse hydrolysate under high gravity fermentation conditions when cultured with ECE and xylose isomerase. The ethanol fermentation is rapid and leads to economically distillable concentrations. The biomass generated can be used for cattle feed as no recombinant organisms are utilized. The self-sufficient generating aspects of this process make it amenable to remote locates prevalent to sugarcane plantations. It would be anticipated that the capability to generate liquid fuel in a remote location could displace the currently common practice of simply burning the waste sugarcane bagasse.

and 55 g/L xylose). (3) Two-stage fermentation process diagram. (4) Effects of ECE and xylose isomerase on ethanol productivity in reagent-grade sugar fermentations.

References

1. Chen, Y.L. Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: A systematic review. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 581–597. [CrossRef] [PubMed]

2. Kumar, D.; Murthy, G.S. Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* **2011**, *4*, 27–45. [CrossRef] [PubMed]

3. Olson, D.G.; McBride, J.E.; Shaw, A.J.; Lynd, L.R. Recent progress in consolidated bioprocessing. *Curr. Opin. Biotechnol.* **2012**, *23*, 396–405. [CrossRef] [PubMed]

4. Himmel, M.E.; Ding, S.Y.; Johnson, D.K.; Adney, W.S.; Nimlos, M.R.; Brady, J.W.; Foust, T.D. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science* **2007**, *315*, 804–807. [CrossRef]

5. Margeot, A.; Hahn-Hagerdal, B.; Edlund, M.; Slade, R.; Monot, F. New improvements for lignocellulosic ethanol. *Curr. Opin. Biotechnol.* **2009**, *20*, 372–380. [CrossRef]

6. Prior, B.A.; Kilian, S.G.; Preez, J.C.d. Fermentation of D-xylose by the yeasts *Candida shehatae* and *Pichia stipitis*. *Process Biochem.* **1989**, *24*, 21–32.

7. Chen, R.; Dou, J. Biofuels and bio-based chemicals from lignocellulose: Metabolic engineering strategies in strain development. *Biotechnol. Lett.* **2016**, *38*, 213–221. [CrossRef]

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. [CrossRef]

9. Okuno, M.; Kajitani, R.; Ryusui, R.; Morimoto, H.; Kodama, Y.; Itoh, T. Next-generation sequencing analysis of lager brewing yeast strains reveals the evolutionary history of interspecies hybridization. *DNA Res.* **2016**, *23*, 67–80. [CrossRef]

10. Quain, D.E. Yeast Genetics in Brewing: New Insights and Opportunies. In *Brewing: New Technologies*; Bamforth, C.W., Ed.; Woodhead Publishing Limited: Cambridge, England, 2006; pp. 149–166.

11. Dunn, B.; Sherlock, G. Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Res.* **2008**, *18*, 1610–1623. [CrossRef]

12. Saerens, S.M.G.; Duong, C.T.; Nevoigt, E. Genetic improvement of brewer's yeast: Current state, perspectives and limits. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1195–1212. [CrossRef] [PubMed]

13. Goncalves, P.; de Sousa, H.R.; Spencer-Martins, I. FSY1, a novel gene encoding a specific fructose/H$^{(+)}$ symporter in the type strain of *Saccharomyces carlsbergensis*. *J. Bacteriol.* **2000**, *182*, 5628–5630. [CrossRef] [PubMed]

14. 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. [CrossRef] [PubMed]

15. Ladisch, M.R.; Svarczkopf, J.A. Ethanol production and the cost of fermentable sugars from biomass. *Bioresource Technol.* **1991**, *36*, 83–95. [CrossRef]

16. Joelsson, E.; Erdei, B.; Galbe, M.; Wallberg, O. Techno-economic evaluation of integrated first- and second-generation ethanol production from grain and straw. *Biotechnol. Biofuels* **2016**, *9*, 1. [CrossRef]

17. Ingledew, W.M. *The Alcohol Textbook: A Reference for the Beverage, Fuel and Industrial Alcohol Industries*; Nottingham University Press: Nottingham, UK, 2009.

18. Son, C.K.; Pham, T.H.; Bui, K.L.T.; Nguyen, T.T.; Pham, K.D.; Nguyen, H.D.T.; Luong, H.N.; Tu, V.P.; Nguyen, T.H.; Ho, P.H.; et al. Simultaneous liquefaction, saccharification and fermentation at very high gravity of rice at pilot scale for potable ethanol production and distillers dried grains composition. *Food Bioprod. Process.* **2016**, *98*, 79–85. [CrossRef]

19. De Bari, I.; Cuna, D.; Di Matteo, V.; Liuzzi, F. Bioethanol production from steam-pretreated corn stover through an isomerase mediated process. *New Biotechol.* **2014**, *31*, 185–195. [CrossRef]

20. Yuan, D.W.; Rao, K.; Relue, P.; Varanasi, S. Fermentation of biomass sugars to ethanol using native industrial yeast strains. *Bioresource Technol.* **2011**, *102*, 3246–3253. [CrossRef]

21. Wei, S.; Bai, P.; Liu, Y.; Yang, M.; Ma, J.; Hou, J.; Liu, W.; Bao, X.; Shen, Y. A Thi2p Regulatory Network Controls the Post-glucose Effect of Xylose Utilization in *Saccharomyces cerevisiae*. *Front. Microbiol.* **2019**, *10*. [CrossRef]

22. Niu, Y.; Wu, L.; Shen, Y.; Zhao, J.; Zhang, J.; Yi, Y.; Li, H.; Bao, X. Coexpression of beta-xylosidase and xylose isomerase in *Saccharomyces cerevisiae* improves the efficiency of saccharification and fermentation from xylo-oligosaccharides. *Cellulose* **2019**, *26*, 7923–7937. [CrossRef]

23. Zhang, M.-M.; Xiong, L.; Tang, Y.-J.; Mehmood, M.A.; Zhao, Z.K.; Bai, F.-W.; Zhao, X.-Q. Enhanced acetic acid stress tolerance and ethanol production in *Saccharomyces cerevisiae* by modulating expression of the de novo purine biosynthesis genes. *Biotechnol. Biofuels* **2019**, *12*, 116. [CrossRef] [PubMed]

24. Bera, A.K.; Ho, N.W.Y.; Khan, A.; Sedlak, M. A genetic overhaul of *Saccharomyces cerevisiae* 424A(LNH-ST) to improve xylose fermentation. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 617–626. [CrossRef] [PubMed]

25. Kricka, W.; James, T.C.; Fitzpatrick, J.; Bond, U. Engineering *Saccharomyces pastorianus* for the co-utilisation of xylose and cellulose from biomass. *Microb. Cell Fact.* **2015**, *14*, 61. [CrossRef] [PubMed]

26. Ko, J.K.; Um, Y.; Woo, H.M.; Kim, K.H.; Lee, S.-M. Ethanol production from lignocellulosic hydrolysates using engineered *Saccharomyces cerevisiae* harboring xylose isomerase-based pathway. *Bioresource Technol.* **2016**, *209*, 290–296. [CrossRef] [PubMed]

27. Moyses, D.N.; Branco Reis, V.C.; Moreira de Almeida, J.R.; Pepe de Moraes, L.M.; Goncalves Torres, F.A. Xylose fermentation by *Saccharomyces cerevisiae*: Challenges and prospects. *Int. J. Mol. Sci.* **2016**, *17*, 207. [CrossRef] [PubMed]

28. Zhao, W.; Zhao, F.; Zhang, S.; Gong, Q.; Chen, G. Ethanol production by simultaneous saccharification and cofermentation of pretreated corn stalk. *J. Basic Microbiol.* **2019**, *59*, 744–753. [CrossRef]

29. Li, Y.-C.; Gou, Z.-X.; Zhang, Y.; Xia, Z.-Y.; Tang, Y.-Q.; Kida, K. Inhibitor tolerance of a recombinant flocculating industrial *Saccharomyces cerevisiae* strain during glucose and xylose co-fermentation. *Braz. J. Microbiol.* **2017**, *48*, 791–800. [CrossRef]

30. Ha, S.J.; Galazka, J.M.; Kim, S.R.; Choi, J.H.; Yang, X.M.; Seo, J.H.; Glass, N.L.; Cate, J.H.D.; Jin, Y.S. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 504–509. [CrossRef]

31. Liu, T.; Huang, S.; Geng, A. Recombinant Diploid *Saccharomyces cerevisiae* Strain Development for Rapid Glucose and Xylose Co-Fermentation. *Fermentation-Basel* **2018**, *4*, 59. [CrossRef]

32. Wang, X.; Yang, J.; Yang, S.; Jiang, Y. Unraveling the genetic basis of fast L-arabinose consumption on top of recombinant xylose-fermenting Saccharomyces cerevisiae. *Biotechnol. Bioeng.* **2018**. [CrossRef]

33. Jayakody, L.N.; Turner, T.L.; Yun, E.J.; Kong, I.I.; Liu, J.-J.; Jin, Y.-S. Expression of Gre2p improves tolerance of engineered xylose-fermenting Saccharomyces cerevisiae to glycolaldehyde under xylose metabolism. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 8121–8133. [CrossRef] [PubMed]

34. Chen, Y.; Wu, Y.; Zhu, B.; Zhang, G.; Wei, N. Co-fermentation of cellobiose and xylose by mixed culture of recombinant Saccharomyces cerevisiae and kinetic modeling. *PLoS ONE* **2018**, *13*. [CrossRef] [PubMed]

35. Mokomele, T.; Sousa, L.D.C.; Balan, V.; van Rensburg, E.; Dale, B.E.; Gorgens, J.F. Ethanol production potential from AFEX (TM) and steam-exploded sugarcane residues for sugarcane biorefineries. *Biotechnol. Biofuels* **2018**, *11*, 127. [CrossRef] [PubMed]

36. Ko, J.K.; Jung, J.H.; Altpeter, F.; Kannan, B.; Kim, H.E.; Kim, K.H.; Alper, H.S.; Um, Y.; Lee, S.-M. Largely enhanced bioethanol production through the combined use of lignin-modified sugarcane and xylose fermenting yeast strain. *Bioresource Technol.* **2018**, *256*, 312–320. [CrossRef] [PubMed]

37. 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. [CrossRef]

38. Sharma, S.S.; Blattner, F.R.; Harcum, S.W. Recombinant protein production in an *Escherichia coli* reduced genome strain. *Metab. Eng.* **2007**, *9*, 133–141. [CrossRef] [PubMed]

39. 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. [CrossRef]

40. 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. [CrossRef]

41. Akesson, M.; Hagander, P.; Axelsson, J.P. A probing feeding strategy for *Escherichia coli* cultures. *Biotechnol. Tech.* **1999**, *13*, 523–528. [CrossRef]

42. 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. [CrossRef]

43. 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*. [CrossRef]

44. 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, DC, USA, 1996; Volume 1, pp. 13–16.

45. Bremer, H.; Dennis, P.P. Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate. 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, DC, USA, 1996; Volume 2, pp. 1553–1569.

46. 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. [CrossRef] [PubMed]

47. Jain, A.; Wei, Y.; Tietje, A. Biochemical conversion of sugarcane bagasse into bioproducts. *Biomass Bioenergy* **2016**, *93*, 227–242. [CrossRef]

48. Jain, A.; Walker, T.H. Pretreatment Composition for Biomass Conversion Process. U.S. Patent 20150087030A1, 26 March 2015.

49. Miller, K.P. Biofuel Ethanol Production by Saccharomyces bayanus, the Champagne Yeast. Master's Thesis, Clemson University, Clemson, SC, USA, 2010.

50. Ingledew, W.M. Alcohol production by Saccharomyces cerevisiae: A yeast primer. In *The Alcohol Textbook*, 3rd ed.; Nottingham University Press: Nottingham, UK, 1999; pp. 49–86.

51. Smith, J.; van Rensburg, E.; Görgens, J.F. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. *BMC Biotechnol.* **2014**, *14*, 1–17. [CrossRef]

52. Kobayashi, Y.; Sahara, T.; Ohgiya, S.; Kamagata, Y.; Fujimori, K.E. Systematic optimization of gene expression of pentose phosphate pathway enhances ethanol production from a glucose/xylose mixed medium in a recombinant Saccharomyces cerevisiae. *Amb Express* **2018**, *8*, 139. [CrossRef]

53. Puligundla, P.; Smogrovicova, D.; Mok, C.; Obulam, V.S.R. A review of recent advances in high gravity ethanol fermentation. *Renew. Energy* **2019**, *133*, 1366–1379. [CrossRef]

54. Qureshi, A.S.; Zhang, J.; Bao, J. High ethanol fermentation performance of the dry dilute acid pretreated corn stover by an evolutionarily adapted *Saccharomyces cerevisiae* strain. *Bioresource Technol.* **2015**, *189*, 399–404. [CrossRef]

55. Zhao, X.; Moates, G.K.; Elliston, A.; Wilson, D.R.; Coleman, M.J.; Waldron, K.W. Simultaneous saccharification and fermentation of steam exploded duckweed: Improvement of the ethanol yield by increasing yeast titre. *Bioresource Technol.* **2015**, *194*, 263–269. [CrossRef]

56. Demeke, M.M.; Dietz, H.; Li, Y.; Foulquié-Moreno, M.R.; Mutturi, S.; Deprez, S.; Den Abt, T.; Bonini, B.M.; Lidén, G.; Dumortier, F.; et al. Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. *Biotechnol. Biofuels* **2013**, *6*. [CrossRef]

57. Casey, G.P.; Magnus, C.A.; Ingledew, W.M. High-Gravity Brewing Effects Of Nutrition on Yeast Composition, Fermentative Ability, and Alcohol Production. *Appl. Environ. Microbiol.* **1984**, *48*, 639–646. [CrossRef]

58. Rao, K.; Chelikani, S.; Relue, P.; Varanasi, S. A novel technique that enables efficient conduct of simultaneous isomerization and fermentation (SIF) of xylose. *Appl. Biochem. Biotechnol.* **2008**, *146*, 101–117. [CrossRef] [PubMed]

59. Wang, W.; Wang, Q.; Tan, X.; Qi, W.; Yu, Q.; Zhou, G.; Zhuang, X.; Yuan, Z. High conversion of sugarcane bagasse into monosaccharides based on sodium hydroxide pretreatment at low water consumption and wastewater generation. *Bioresource Technol.* **2016**, *218*, 1230–1236. [CrossRef] [PubMed]