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A Chemical-Free Pretreatment for Biosynthesis of Bioethanol and Lipids from Lignocellulosic Biomass: An Industrially Relevant 2G Biorefinery Approach

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Abstract: A wide range of inorganic and organic chemicals are used during the pretreatment and enzymatic hydrolysis of lignocellulosic biomass to produce biofuels. Developing an industrially relevant 2G biorefinery process using such chemicals is challenging and requires more unit operations for downstream processing. A sustainable process has been developed to achieve industrially relevant titers of bioethanol with significant ethanol yield. The pretreatment of sorghum biomass was performed by a continuous pilot-scale hydrothermal reactor followed by disk milling. Enzymatic hydrolysis was performed without washing the pretreated biomass. Moreover, citrate buffer strength was reduced to 100-fold (50 mM to 0.5 mM) during the enzymatic hydrolysis. Enzymatic hydrolysis at 0.5 mM citrate buffer strength showed that significant sugar concentrations of 222 ± 2.3 to 241 ± 2.3 g/L (glucose + xylose) were attained at higher solids loadings of 50 to 60% (*w*/*v*). Furthermore, hydrolysates were fermented to produce bioethanol using two different xylose-fermenting *Saccharomyces cerevisiae* strains and a co-culture of xylose-fermenting and non-GMO yeast cultures. Bioethanol titer of 81.7 g/L was achieved with an ethanol yield of 0.48 g_p/g_s. Additionally, lipids were produced using the oleaginous yeast *Rhodosporidium toruloides*, yielding 13.2 g/L lipids with cellular lipid accumulation of 38.5% *w*/*w* from 100 g/L of sugar concentration. In summary, reducing the strength of the citrate buffer during enzymatic hydrolysis and omitting inorganic chemicals from the pretreatment process enhances the fermentability of hydrolysates and can also reduce operating costs.

Keywords: hydrothermal pretreatment; bioenergy sorghum; reduced citrate buffer; enzyme hydrolysis; fermentation

Citation: Deshavath, N.N.; Dien, B.S.; Slininger, P.J.; Jin, Y.-S.; Singh, V. A Chemical-Free Pretreatment for Biosynthesis of Bioethanol and Lipids from Lignocellulosic Biomass: An Industrially Relevant 2G Biorefinery Approach. *Fermentation* **2023**, *9*, 5. https://doi.org/10.3390/fermentation9010005

Academic Editors: Yaohua Zhong and Yinbo Qu

Received: 1 December 2022
Revised: 13 December 2022
Accepted: 19 December 2022
Published: 21 December 2022

1. Introduction

Conversion of lignocellulosic biomass into biofuels and bioproducts has gained momentum in scientific and industrial research as one option for partially mitigating climate change [1–3]. Biochemical technology is a leading process option for the production of advanced biofuels. Biochemical processing requires the pretreatment of biomass to open up the plant cell wall followed by enzymatic conversion of cellulose and hemicellulose to sugars and microbial conversion of sugars to the targeted biofuel (e.g., ethanol) [4]. Hydrothermal-based pretreatments are a leading approach for commercialization because they avoid the addition of harmful chemical catalysts (acids or alkalis) that add operating costs and complicate downstream processing. In this regard, we developed a two-stage pretreatment process whereby biomass is treated at 190 °C for 10 min at 50% moisture

followed by disc milling [5]. Disc milling further opens up the cellulose fibers and increases enzymatic sugar yields at relatively low severity pretreatment conditions. This process has since been scaled up with the use of a continuous steam explosion reactor using bioenergy sorghum as the feedstock [6]. A high-solids enzymatic hydrolysis (50% solids loading) achieved a sugar concentration of 231.8 g/L, which is 65% of the theoretical concentration [7].

Another major advantage of the two-stage hydrothermal and mechanical pretreatment process is that it minimizes the formation of fermentation inhibitors. Furfural, 5-hydroxymethylfurfural (5-HMF), levulinic acid, and formic acid can be formed during pretreatment of lignocellulosic biomass under acidic conditions at moderate- to high-reaction temperatures [8]. Furfural is a degradation product of xylose and arabinose and 5-HMF is a degradation product of glucose and fructose. Further decomposition of 5-HMF forms levulinic acid and formic acid, whereas furfural forms only formic acid. Our process minimizes the formation of these products because it operates at a lower severity and, without acid, it limits the formation of sugar decomposition products. However, constituent inhibitory chemicals are still released, which include acetic acid, glucuronic acid, ferulic acid, vanillic acid, coumaric acid, and syringaldehyde from the lignin and hemicellulose fractions [9–12]. The composition of lignocellulosic biomass constituents varies based on the type of species, biological origin, growth conditions, period of harvesting, and storage time [12,13].

Sugars prepared from steam explosion/disc milling have been used to produce both ethanol and single-cell lipids (e.g., triacylglycerols) [14]. The latter are good candidates for the production of biodiesel, green diesel, and sustainable jet fuel based on fatty acid composition [15]. A major barrier to microbial production of biofuels is the mixed sugars found in hydrolysates. Following pretreatment and enzymatic hydrolysis of herbaceous biomass, as much as 40% of the released sugars is xylose. Xylose is problematic because it is not fermented by Distillers' yeast [16]. In this study, *Saccharomyces* strains are evaluated that have been engineered to ferment xylose [17]. Moreover, the oleaginous yeast selected for this study metabolizes glucose, xylose, and other biomass-released sugars and has proven to be robust for growth in hydrolysates [14].

Enzymatic hydrolysis of pretreated biomass into monosaccharides requires the use of cellulases, hemicellulases, and auxiliary enzymes. The use of enzymes requires controlling pH within a narrow range, which is typically accomplished by using a 50 mM citrate buffer solution. This consumes 19.2 kg of citrate per ton of biomass. Therefore, adding buffer inflates processing cost and at 50 mM concentration of citrate acts as a fermentation inhibitor [18,19]. In our earlier research, dilute sulfuric acid pretreated and delignified sorghum biomass were enzymatically hydrolyzed at reduced citrate buffer strengths (5 mM and 0.5 mM), resulting in equivalent cellulose to glucose conversion efficiencies at all citrate buffer strengths (50 mM, 5 mM, and 0.5 mM) [18,19]. However, the major limitation of these studies was the washing of pretreated and delignified biomass before the enzymatic hydrolysis, which necessitates a significant quantity of water. When it is scaled up to the pilot level, it frequently becomes crucial. Additionally, wash water comprising inorganic acids, sulfate ions, and sodium salts needs to be treated in a specialized wastewater treatment section [20]. Another work employed dilute sulfuric acid pretreatment of pine biomass and performed enzymatic hydrolysis at 5 mM citrate buffer without washing, leading to no cellulose hydrolysis [21]. This study also stated that the presence of acetic acid, formic acid, furfural, and 5-HMF did not affect the cellulose hydrolysis of unwashed pretreated pine biomass when the enzymatic hydrolysis was carried out at 50 mM citrate buffer strength; however, cellulose hydrolysis could still occur in unwashed biomass [21].

In light of the previous research, the possibility of reducing the citrate concentration by 10- and 100-fold is investigated for bioenergy sorghum processed using two-stage hydrothermal mechanical pretreatment without washing. Sugar yields were compared for enzymatic hydrolysis of pretreated biomass using 0.5 mM, 5.0 mM, and 50 mM citrate buffer for pH control. Additionally, the hydrolysis sugars were subsequently evaluated

for production of bioethanol and lipids. Ethanol fermentations were evaluated using two *Saccharomyces* yeast strains engineered to ferment xylose. One was a commercially available strain and the second a laboratory-developed strain suited for industrial use because it does not contain antibiotic resistance genes. Ethanol fermentations were also run using a co-culture of a popular non-GMO *Saccharomyces* yeast strain (Ethanol Red) and a GMO commercial strain, which could utilize xylose. Lipid production cultures used a *Rhodosporidium toruloides* strain determined earlier to work well with this feedstock.

2. Materials and Methods

2.1. Feedstock

Bioenergy sorghum was harvested from the Energy Farm (University of Illinois, Urbana, IL, USA) and dried at ambient temperature to <10% (*w*/*w*) moisture. The biomass was chopped (to pieces approximately 3–4 cm long) and ground with a hammer mill to pass through a 3 mm sieve. Ground biomass was stored at 4 °C. The biomass contained (%*w*/*w*, odb): 12% extractives, 39% cellulose, 22% xylan, and 16% lignin [7].

2.2. Pretreatment

A pilot scale continuous hydrothermal reactor system (SüPR●2G Reactors, AdvanceBio system LLC., Milford, OH, USA) was used to pretreat bioenergy sorghum biomass at 190 °C for 10 min [6]. This optimal reaction condition was attained from our earlier research, where the maximum sugar yields were attained during enzymatic hydrolysis [6]. The moisture content of lignocellulosic biomass was adjusted to 50% (*w*/*w*) before the hydrothermal pretreatment. The pretreated biomass was dried at 46 ± 3 °C in a conventional tray drier until the moisture content was ≤20%. The pretreated biomass was disc milled to further reduce particle size and increase the surface area [6].

Compositional Analysis of Hydrothermal Pretreated Biomass

Pretreated biomass was analyzed for composition using modified National Renewable Energy Laboratory (NREL) protocols. The biomass was extracted with water and ethanol, and the extractives were analyzed for xylo- and cello-oligomers and monomers. The following equations were used for the determination of moisture (Equation (1)) and total solids (Equation (2)), oven dry weight (ODW) (Equation (3)), and extractive (Equation (4)) contents of hydrothermally pretreated biomass.

$$Moisture\ (\%) = \left[\frac{(W_1 - W_2)}{W_1}\right] \times 100 \quad (1)$$

W_1 is the weight of air-dried biomass and W_2 is the weight of biomass after drying at 105 °C for 4 h.

$$Total\ solids\ (\%) = 100 - Moisture\ (\%) \quad (2)$$

$$ODW\ (g) = \left[\frac{W_1 \times \%Total\ solids}{100}\right] \quad (3)$$

$$Extractives\ (\%) = \left[\frac{\left(W_i - W_f\right)}{W_i}\right] \times \%Total\ solids \quad (4)$$

W_i and W_f are the weights of biomass before and after Soxhlet extraction, respectively.

The water-soluble extractive was directly hydrolyzed with cellulases and hemicellulase. For the ethanol extractive, the ethanol was evaporated using a rotary evaporator and the solids reconstituted using water. Cellulase (80 mg protein/g cellulose) and hemicellulase (60 mg protein/g of xylan), based on cellulose and xylan contents of the initial biomass, were applied to hydrolyze cello-and xylo-oligomers in the extractives. Enzymatic hydrolysis was conducted in 50 mM citrate buffer strength at 50 °C for 24 h in 250 mL screw cap conical flasks shaken at 150 rpm. The percentage of cellulose and xylan in the

extractives can be calculated by Equation (5) along with anhydrous correction (Equation (6)).

$$S_{ext.}(\%) = \left[\frac{Sugar\ (mg/mL)\ by\ HPLC \times V_{eh}\ (mL) \times \frac{1\ g}{1000\ mg}}{ODW_{sample}\ (g)}\right] \times 100 \quad (5)$$

$S_{ext.}$ is the sugars (cellobiose or glucose or xylose or arabinose) in the water and ethanol extractives. V_{eh} is the total volume of the enzymatic hydrolysate of extractives.

$$Pfs_{ext.}\ (\%) = S_{ext.}\ (\%) \times Anhydro\ correction \quad (6)$$

$Pfs_{ext.}$ is the polymeric form of sugars (cellulose and xylan/hemicellulose) in the water and ethanol extractives. The anhydrous correction for glucose and pentose sugars (xylose and arabinose) are 0.90 (162/180) and 0.88 (132/150), respectively. The acetic acid to acetate conversion factor is 0.983 (59/60) [22].

The extractive free biomass was further reprocessed using a two-step acid hydrolysis (300 mg on ODW) for structural carbohydrates analysis. The percent of cellulose, xylan, and acid insoluble lignin in the extractive free biomass were determined as follows (Equations (7)–(11)).

$$S_{ext.free}\ (\%) = \left[\frac{Sugar\ (mg/mL)\ by\ HPLC \times V_{filtrate}\ (mL) \times \frac{1\ g}{1000\ mg}}{ODW_{sample}\ (g)}\right] \times 100 \quad (7)$$

$$Pfs_{ext.free}\ (\%) = \left(\frac{Sugar_{ext.free}\ (\%)}{Sugar_{correction}}\right) \times Anhydro\ correction \quad (8)$$

$$Pfs_{Including\ extractives}\ (\%) = (\%pfs_{ext.free}\ ) \times \left(\frac{100 - \%Extractive}{100}\right) \quad (9)$$

$$AIL_{ext.free}\ (\%) = \left[\frac{AIR - Ash}{ODW_{sample}}\right] \times 100 \quad (10)$$

$$AIL_{Including\ extractives}(\%) = (\%AIL_{ext.free}\ ) \times \left(\frac{100 - \%Extractive}{100}\right) \quad (11)$$

Finally, summation Equations (6) and (9) provide the cellulose and xylan contents of hydrothermal pretreated biomass.

$$Structural\ carbohydrate\ (\%) = \%Pfs_{ext.} + \%Pfs_{Including\ extracives} \quad (12)$$

2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in 500 mL screw cap conical flasks containing a final composition of 36 g of pretreated biomass (on dry weight basis) per 60 mL of liquid medium. Enzymatic hydrolysis was conducted as a fed batch process to achieve high solids loading of pretreated biomass and concentrated sugars. Citrate buffer was kept at 0.5 mM, 5 mM, and 50 mM. Initially, 20% (w/v) pretreated biomass was loaded into the flask and then solids loading was increased to 30% (2.8 mL of 25% PEG 4000 added), 40%, 50%, and 60% at 6 h, 12 h, 24 h, and 48 h, respectively. For quantification of sugars, a 0.5 mL aliquot was collected from the hydrolysate at every time interval before every 10% increment in solids loadings from 20% to 60%. Biocatalysts, cellulase NS22257, and hemicellulase NS22244 (Novozymes North America, Inc., Franklinton, NC, USA) were used at 60 mg of cellulase protein/g of cellulose and 20 mg of hemicellulase protein/g of hemicellulose. The protein concentration of cellulase and hemicellulase was determined by bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific™, Waltham, MA, USA). Samples were hydrolyzed at 50 °C while mixing at 160 rpm for 72 h, with periodic sampling for sugars. Based on these preliminary results, enzymatic hydrolysis was scaled

10× to 300 g of pretreated biomass in 600 mL buffer using a 2 L capacity screw cap conical flask.

After the enzymatic hydrolysis, the slurry was centrifuged to remove solids and the pH of the hydrolysate was adjusted to 5.6 with 5N NaOH. Hydrolysates used for fermentations were filter sterilized.

Cellulose and xylan yields were calculated based on the following equation:

$$Y_{Gul./Xyl.}\ (\%) = \left[\frac{C_{sugar} \times V_L \times CF}{S_C \times S_L}\right] \times 100 \quad (13)$$

where $Y_{Gul\cdot/Xyl\cdot}$ is the yield of glucose and xylose; C_{sugar} [g/L] is the concentration of glucose or xylose; V_L [L] is the liquid volume of enzymatic hydrolysis medium (enzymes, moisture content of pretreated biomass, 25% PEG 4000 and citrate buffer); CF is the conversion factor (1.10) to account for relative change in volume during hydrolysis from water consumed by hydrolysis and expansion from released sugars going into solution, and expansion from released sugars is based on standard dextrose specific gravity; S_C [g/g] is the structural carbohydrate (cellulose and xylan) content of pretreated biomass, and S_L is the percentage of solids loading (pretreated biomass on dry weight basis) during the enzymatic hydrolysis.

2.4. Fermentation

2.4.1. Microorganisms and Seed Culture Preparation

Ethanol Red (Fermentis-Lesaffre Yeast Corporation, Milwaukee, WI, USA), *S. cerevisiae* CT2 pro (GMO glucose and xylose fermenting yeast) [17], and a commercial GMO xylose-fermenting *Saccharomyces* were used for ethanol fermentations and *Rhodosporidium toruloides* Y-6987 (ARS Culture Collection, Peoria, IL, USA) was used for lipid production. Yeast strains were maintained on YPD agar medium, which contains (g/L: 10, yeast extract; 20, peptone; 20, dextrose; and 20, agar). Yeast strains were grown at 30 °C and pH 5.6.

The seed cultures were grown in 20 mL of YPDX medium in a 100 mL screw cap conical flask consisting of YP supplemented with 12 g/L glucose and 8 g/L xylose [23]. Seed cultures were inoculated with a colony of the commercial GMO xylose fermenting yeast strain or CT2 pro and grown at 30 °C for 18 h with 140 rpm and the initial pH of the medium was adjusted to 5.6. Microbial cells were harvested by centrifugation at 8000 rpm for 10 min and pellets were resuspended in sterile distilled water and used to inoculate the yeast ethanol cultures.

Ethanol red yeast (2 g dry) was dispersed in 20 mL of sterile distilled water and activated by incubating at 30 °C for 20 min while mixing at 100 rpm. The culture was centrifuged, and the yeast resuspended in an equal volume of sterile-distilled water to serve as an inoculum.

The *R. toruloides* pre-seed culture was inoculated by transferring a single colony from plate into 2 mL of YPD (10 g/L, yeast extract; 20 g/L peptone; and 50 g/L glucose) in a 16 mL snap tube and incubated at 28 °C for 18 h at 250 rpm. Seed culture media was prepared from hydrolysates containing either 50 mM or 0.5 mM citrate buffer. The seed medium contained per 100 mL: 21.2 mL hydrolysate, 10 mL mineral stock, 6.7 mL nitrogen stock, and 6.21 mL either 0.5 or 50 mM citrate solution, adjusted to pH 6.0. The nitrogen stock (4 g yeast extract and 0.5 g $(NH_4)_2SO_4$ in 100 mL distilled water) and 10× mineral solution (1 g KH_2PO_4 and 1 g $MgSO_4$ (or 2.05 g $MgSO_4$ $7H_2O$) in 100 mL distilled water) were filter sterilized. The pre-seed culture was transferred to the 25 mL seed culture (5% v/v) in 125 mL baffled flasks and incubated for 48 h at 28 °C while mixing at 250 rpm. Cells were harvested by centrifugation and concentrated in sterile-distilled water to an A_{600} ~50.

2.4.2. Fermentation of Enzymatic Hydrolysates for Bioethanol Production

Enzymatic hydrolysates derived from 50 mM, 5 mM, and 0.5 mM citrate buffer strengths at 50% (w/v) solids loading were fermented by ethanologic yeast to evaluate the effect of citrate buffer on fermentation efficiency. Fermentation experiments were con-

ducted in 250 mL screw cap Erlenmeyer flasks which contained 94 mL of filter-sterilized hydrolysate, 4 mL of 25× YP nutrient solution, and 2 mL of seed culture, which provided an initial A_{600} ~1. The initial pH of the fermentation culture was adjusted to 5.6 and incubated at 30 °C for 144 h while mixing at 150 rpm.

Co-culture fermentations used a commercial *S.* cerevisiae strain engineered for xylose metabolism and a non-GMO industrial *Saccharomyces* yeast (Ethanol Red) to enhance the ethanol titer and sugar (especially xylose) consumption. Enzymatic hydrolysates produced using 50 mM, 5 mM, and 0.5 mM citrate buffer strength at 60% (*w*/*v*) solids loading were used in the co-culture fermentations. The fermentation cultures were inoculated with 1 mL of Ethanol Red and 1 mL of recombinant *S. cerevisiae* seed cultures so as to achieve a beginning A_{600} of ~1 with equal contributions of A_{600} from each yeast. Samples are withdrawn at periodic intervals and filtered through a 0.2 µm syringe filter before HPLC analysis for quantification of sugars and ethanol.

2.4.3. Fermentation of Enzymatic Hydrolysates for Microbial Lipids Production

Lipid production micro cultures were run in 48-well flower plates with continuous biomass (e.g., backscatter) measurement using a Biolector (Beckman Coulter, Brea, CA, USA). The 0.5 mM and 50 mM buffered hydrolysates (60% solids) were diluted to 75 g/L, 100 g/L, and 125 g/L total sugars after addition of mineral stock, nitrogen stock, and make-up citrate buffer (0.5 or 50 mM). The volume of nitrogen added was adjusted to maintain a constant C:N: 1.0 mL, 1.3 mL, and 1.7 mL for the 75 g/l, 100 g/l, and 125 g/l media, respectively. The media were adjusted to pH 6 by adding 10 N NaOH. Each micro-well was filled with 0.8 mL of medium and inoculated to an A_{600} of 1. The Biolector was set to 28 °C and 800 rpm and run for 96 h. Residual sugar/acetate and yeast lipid concentrations were analyzed by HPLC and sulfo-phospho-vanillin assay [24], respectively.

Batch cultures were conducted in 250 mL baffled flasks filled with 50 mL of cultures and plugged with aluminum caps. The same media was used as described above for the 0.5 mM and 50 mM citrate-buffered hydrolysates diluted to 100 g/L sugars. Cultures were inoculated to an A_{600} of 1 and incubated at 28 °C for 4 days while mixing at 250 rpm. Cultures were sampled periodically for pH, microbial growth, residual sugars and acetate, and yeast lipids as described above.

2.5. Analytical Methods

Sugars (glucose, xylose, and arabinose), fermentative inhibitors (acetic acid, furfural, 5-HMF, levulinic acid, and formic acid) and ethanol were quantified using a Waters HPLC system (Waters e2695 Separation Module, Waters Corporation, Milford, MA, USA) equipped with 2414 refractive index detector. Samples were eluted through an Aminex HPX-87H column (300 × 7.8 mm, 9µm particle size; Bio-Rad Laboratories, Hercules, CA, USA) at 65 °C and 5 mM H_2SO_4 at 0.6 mL/min flow rate. The RI detector was maintained at 30 °C.

Enzymatic hydrolysis and lipid production experiments were conducted in triplicate. Bioethanol production experiments were conducted in duplicate. Mean values are reported along with the standard deviation.

3. Result and Discussion

3.1. Effect of Hydrothermal Pretreatment Determined by Compositional Analysis

Bioenergy sorghum was pretreated with hydrothermal/disc milling to prepare it for enzymatic hydrolysis. Hydrothermal pretreatment followed by disc milling is highly effective at modifying the physiochemistry of the cell wall to greatly reduce biomass recalcitrance [25,26]. It especially impacts xylan because this polymer disassociates at a relatively low temperature, 140 °C to 220 °C [27]. The pretreated biomass (liquid and solids) contained: 44.97% glucans and 22.2% xylan, which is in close agreement with the untreated biomass composition (39% glucans and 22% xylan) [7]. The pretreated biomass sample was subsequently extracted with water and ethanol and the extracts and residual solids were analyzed for composition. This allowed for determining soluble

saccharides (extracts) and cellulose and xylan (solids) (Table 1). These results indicated that 76.4% of the xylan and 28.4% of the cellulose were solubilized—almost all in the form of oligosaccharides. Lignin largely remained with the solids. It is notable that the lignin of the unextracted pretreated biomass was much higher (28.8%) than the extracted sample (15.7%), which confirms the importance of extracting the sample to obtain an accurate measure of remaining lignin. In summary, hydrothermal pretreatment hydrolyzed much of the xylan and a considerable amount of the cellulose; however, the overall carbohydrate contents were preserved throughout the process.

Table 1. Composition analysis of hydrothermally pretreated bioenergy sorghum biomass.

Components (%*w*/*w*) *	Soxhlet Extracted	Without Soxhlet Extraction
Extractives	35.5 ± 0.14	
Water		
Cellulose	7.21 ± 0.06 †	
Xylan	12.66 ± 0.03 ‡	
Arabinan	0.69	
Acetic acid	1.08	
Ethanol		
Cellulose	5.56 ± 0.39 †	
Xylan	4.31 ± 0.35 ‡	
Acetic acid	0.44 ± 0.036	
Cellulose	32.2 ± 0.4 †	42.18 ± 0.1
Hemicellulose	8.21	25.07
Xylan	5.23 ± 0.17 ‡	15.9 ± 0.36
Arabinan	1.86 ± 0.012	6.75 ± 1.68
Acetic acid	1.12 ± 0.18	2.42 ± 0.17
Lignin	15.7 ± 1.12	28.8 ± 2.1

* Mean value ± standard deviation. † Addition of values gives the actual content of cellulose in the pretreated biomass. ‡ Addition of values gives the actual content of xylan in the pretreated biomass.

3.2. Enzymatic Hydrolysis

Effect of Citrate Buffer Strength on Enzymatic Hydrolysis

Pretreating biomass at high temperatures in the presence of an acid catalyst releases monosaccharides, which subsequently decompose into furfural, 5-HMF, formic acid, levulinic acid, and other compounds [8,28]. The presence of these compounds at high concentration interferes with enzymatic hydrolysis efficiency, which leads to lower sugar yields [29]. Therefore, enzymatic hydrolysis is generally conducted at high-citrate buffer strength (50 mM) to control pH [21]. Since hydrothermal pretreatment of bioenergy sorghum biomass generates negligible amounts of sugar decomposition products, it was hypothesized that the concentration of citrate can be greatly reduced without impacting sugar yields.

Enzymatic hydrolysis was conducted using 0.5 mM, 5 mM, and 50 mM citrate buffer at solids loadings of 20–60% w/v. Glucose and xylose yields were found to be similar at all citrate buffer strengths and solids loadings (Figure 1a). For example, at 20% w/v solids, at the three different buffer concentrations, glucose yields were 80.08 ± 1.5%–83.27 ± 0.8% and xylose yields were 90.2 ± 2%–90.86 ± 0.45%. At higher solids loadings, sugar yields fell. The average glucose and xylose yields were 81.78 ± 1.5% and 90.6 ± 0.4% at 20% solids and 62.49 ± 0.55% and 73.20 ± 0.63% at 60% solids. While yields decreased, sugar concentrations increased with greater solids (Figure 1b). Total sugar concentrations (glucose + xylose, g/L) were 103.2 ± 1.32, 147.4 ± 1.06, 186.7 ± 0.27, 222.3 ± 2.8, and 241.1 ± 2.35 at 20%, 30%, 40%, 50%, and 60% solids loadings, respectively (Figure 1b). The data were statistically analyzed by ANOVA using Origin software and the corresponding p-values for each solids loading were less than 0.005, which are shown in Table S1.

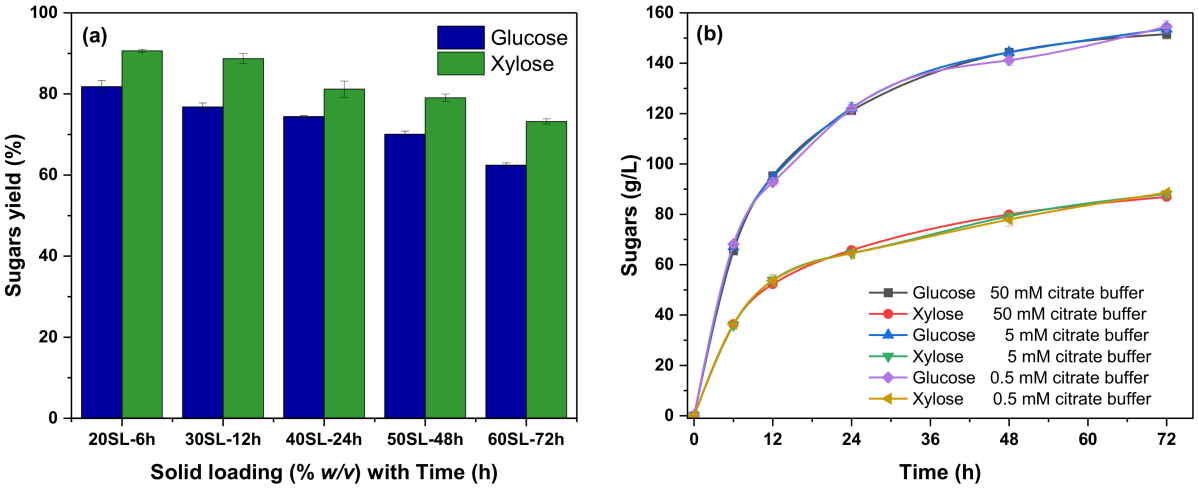

Figure 1. Enzymatic hydrolysis of hydrothermally pretreated biomass at 20% to 60% (*w*/*v*) solids loadings in different citrate buffer strengths: (**a**) glucose and xylose yields; (**b**) concentration of glucose and xylose.

Other studies have also observed that high solids loadings led to decreased sugar yields, primarily because of mass transfer limitations [30]. Lignin is insoluble and, therefore, affects the mixing regime and obstructs the uniform distribution of enzymes. Increased lignin concentration also leads to non-productive binding of enzymes [29,31]. Most pretreatments effectively break down the plant cell wall, but a small percentage of cellulose and xylan are still encapsulated by lignin, which impedes enzyme accessibility. Based on our previous study, 15% of the cellulose and 32% of the xylan was unhydrolyzed even at 10% (*w*/*w*) solids loading [7]. Another study from NREL reported that ~12% cellulose and ~15% xylan remain unconverted even after the dilute acid pretreatment was followed by enzymatic hydrolysis of corn stover [20,32].

Therefore, lowering the citrate concentration from 50 mM to 0.5 mM did not affect sugar yields or titers. This is a superior result to other studies that sought to substitute for sodium citrate buffer. NREL conducted enzymatic hydrolysis of dilute acid pretreated corn stover without sodium citrate buffer but used ammonium hydroxide for adjusting the medium pH between 4.8–5.5, which results in high levels of ammonium salts in stillage, which complicated the treatment of waste water [32]. Several studies used KOH and NaOH to adjust the pH of acid-pretreated biomass for subsequent enzymatic hydrolysis; however, sulfate salts formed and eventually inhibited yeast [33].

The optimal solids loading was determined based on marginal increases in sugar concentration with greater solids. The marginal increases in sugar concentration were 35.6–44.2 g/L up to 50% solids before declining to 18.8 g/L at 60% solids (Figure S1). This led to a lower sugar yield on a per biomass basis at 60% solids. Therefore, the process was scaled 10× for 30% and 50% solids. At 30% solids, the sugar concentration was 147.4 g/L after 24 h with a sugar yield > 77% (Figure 2a,b). The 50% solids loading achieved 215.3 g/L sugars after only 48 h with a yield > 68% (Figure 2a,b). After 48 h, the sugar concentration increased only by 5 g/L (Figure S2). Therefore, the optimal saccharification conditions are 50% solids for 48 h.

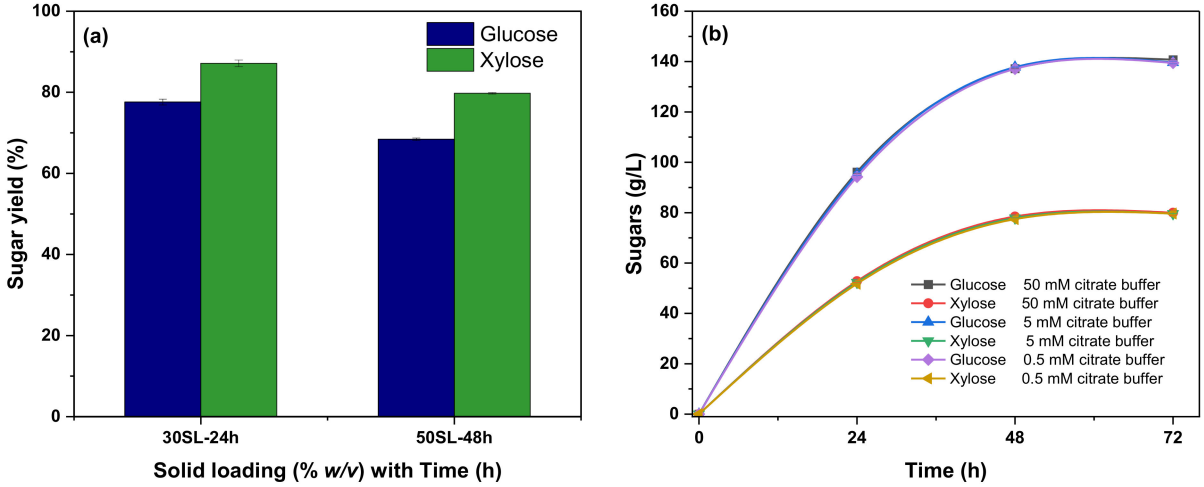

Figure 2. Scale-up study of enzymatic hydrolysis of hydrothermally pretreated biomass at 30% and 50% (*w*/*v*) solids loadings in different citrate buffer strengths: (**a**) glucose and xylose yields; (**b**) concentration of glucose and xylose.

Sugar concentrations were the same for 50% solids hydrolyzed for 48 h using either 0.5 mM, 5 mM, or 50 mM citrate buffer (Table 2). The compositions are notable for their final high-sugar concentration (218 g/L) and low concentrations of furfural (0.6–0.8 g/L) and HMF (0.1–0.3 g/L).

Table 2. Composition of enzymatic hydrolysates at 50% (*w*/*v*) solids loading.

Citrate Buffer (mM)	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Furfural (g/L)	5-HMF (g/L)	Acetic Acid (g/L)	Formic Acid (g/L)	Levulinic Acid (g/L)
50	6.55 ± 0.06	137.2 ± 0.17	78.4 ± 0.51	4.36 ± 0.05	0.06 ± 0.02	0.02 ± 0.007	11.2 ± 0.02	3.55 ± 0.01	2.90 ± 0.01
5	6.76 ± 0.05	137.8 ± 0.17	77.8 ± 0.21	4.39 ± 0.05	0.08 ± 0.01	0.01 ± 0.001	11.4 ± 0.01	3.5 ± 0.01	2.83 ± 0.04
0.5	7.72 ± 0.07	137.1 ± 0.15	77.4 ± 0.14	4.37 ± 0.03	0.08 ± 0.01	0.03 ± 0.001	11.4 ± 0.01	3.61 ± 0.05	2.83 ± 0.01

Mean value ± standard deviation.

From Figures 1a and 2a, considering the yields of glucose (~70%) and xylose (~79%) at 50% (*w*/*v*) solids loading, 315 g of glucose and 175 g of xylose were produced per kg of hydrothermal pretreated bioenergy sorghum biomass. A total of 70% of hydrolysate was recovered through the centrifugation of enzymatic hydrolysis slurry. Therefore, based on the mass balance analysis, 220 g of glucose and 122 g of xylose were recovered from the enzymatic hydrolysis medium. The remaining monomeric sugars in the pellet were extracted by multiple washing with distilled water. All the fractions were pooled to calculate the sugar (in grams) content, accounting for 94 g of glucose and 52 g of xylose. Moreover, 134 g of cellulose and 46 g of xylan were present in the unhydrolyzed biomass. A complete mass balance analysis is shown in Figure 3.

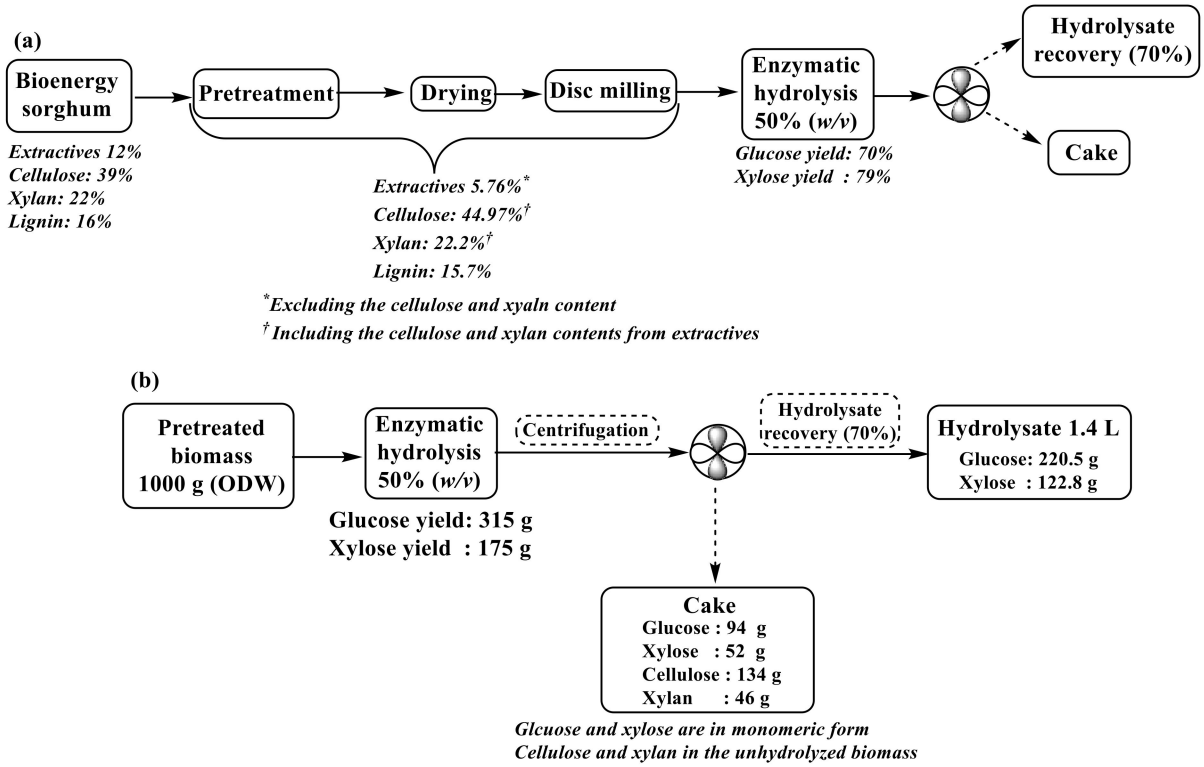

Figure 3. Carbohydrates mass balance analysis of enzymatic hydrolysis at 50% (*w*/*v*) solids loading: (**a**) composition and (**b**) sugar yields.

3.3. Ethanol Fermentation

3.3.1. Effect of Citrate Buffer Strength on Bioethanol Production

Enzymatic hydrolysates prepared using 0.5 mM, 5 mM, and 50 mM citrate buffers were evaluated for ethanol fermentation using two *Saccharomyces* strains engineered for xylose fermentation: a commercially available strain (Figure 4) and a laboratory strain (CT2 Pro, Figure 5). Strain CT2 Pro was specifically engineered for industrial use because it was constructed in such a way as to avoid the use of antibiotic-resistance genes [17]. For both yeast strains, the 50 mM citrate buffered cultures fermented slower than the 0.5 mM or 5 mM buffered cultures. For example, after 48 h, the commercial xylose-fermenting yeast cultures reached ethanol concentrations of 74.4 g/L for the reduced citrate buffer concentrations and only 59.1 g/L for the 50 mM citrate buffered culture (Figure 4a–c).

Despite differences in productivities across buffer strengths, the fermentations achieved similar end titers of ethanol. The commercial xylose-fermenting yeast produced 77.2–81.7 g/L and CT2 Pro 70.4–71.3 g/L of ethanol. Differences between the commercial and CT2 Pro fermentations could be accounted for by differences in xylose consumption. The commercial yeast left 30.4–46.6 g/L xylose unconsumed (Figure 4a–c) and CT2 Pro yeast 49.6–53.23 g/L (Figure 5a–c).

In an attempt to improve xylose consumption, a co-culture of the commercial GMO xylose-fermenting yeast strain and a non-GMO commercial yeast strain (Ethanol Red) was used for the fermentations. Ethanol production and glucose consumption were slower when 50 mM citrate was added compared with 0.5 mM or 5 mM citrate, as was observed for the mono yeast cultures (Figure 6a–c). The co-culture fermentations produced approximately 78.6–80.3 g/L ethanol (Figure 6), which is similar to mono-culture. However, this hydrolysate batch used for the co-culture contained more glucose (141.6 ± 0.5 g/L glucose versus 123 ± 1.5 g/L glucose) and, therefore, actually less xylose was fermented in the co-culture than mono-cultures.

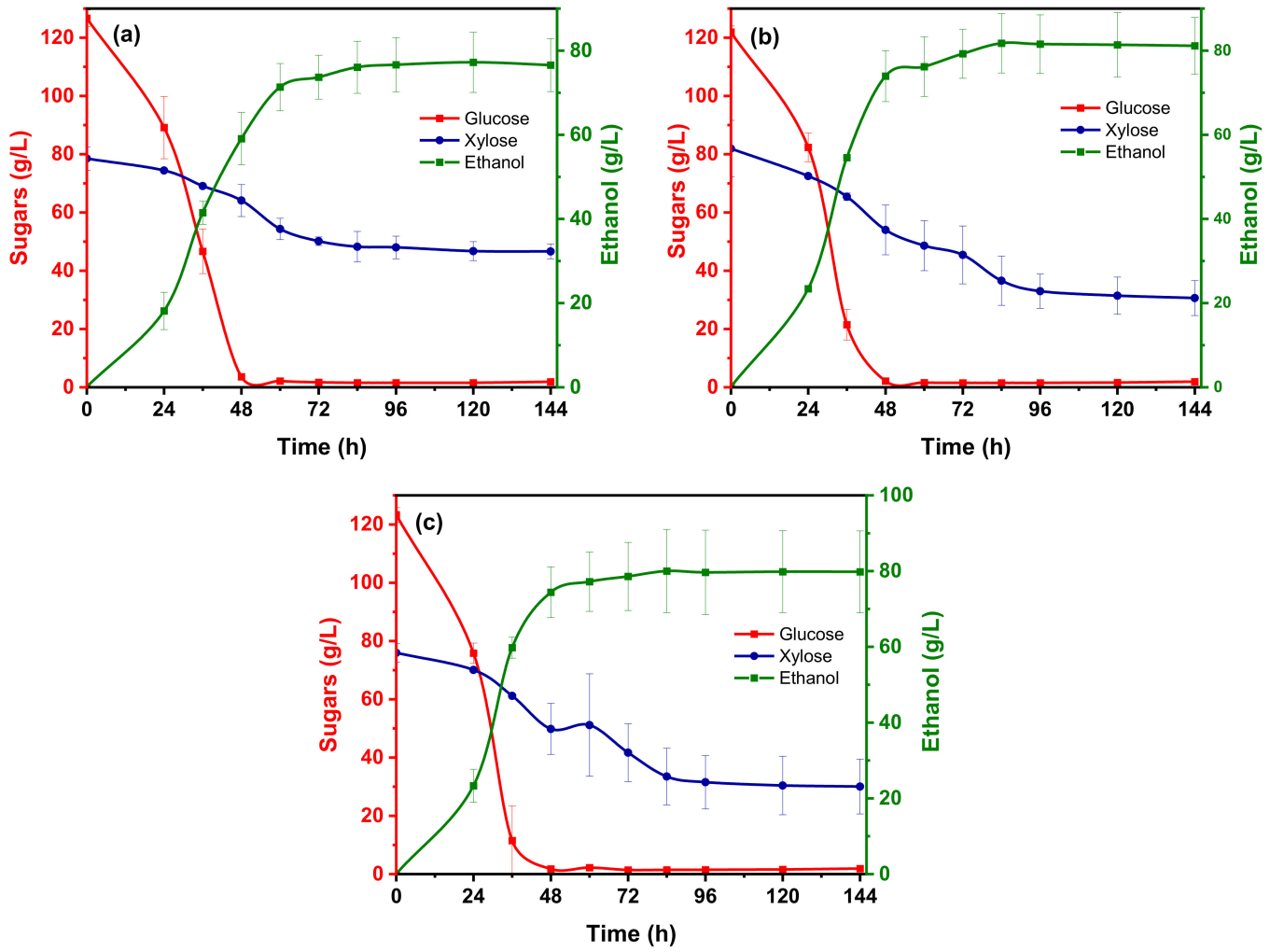

Figure 4. Sugar utilization and bioethanol production profiles of commercial recombinant S. cerevisiae from (**a**) 50 mM citrate buffer, (**b**) 5 mM citrate buffer, and (**c**) 0.5 mM citrate buffer hydrolysates.

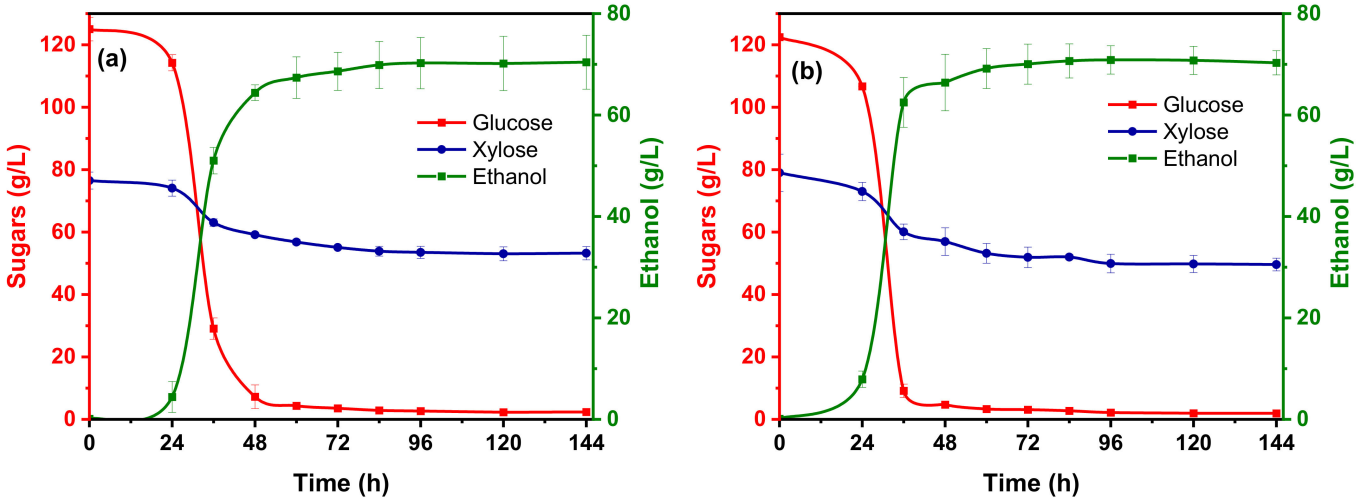

Figure 5. *Cont.*

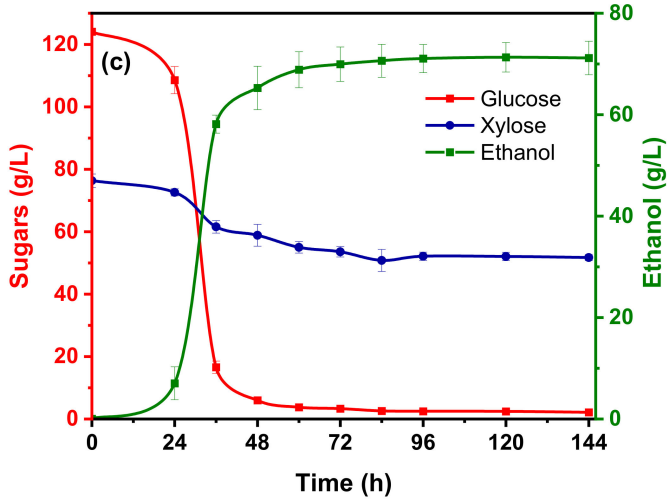

Figure 5. Sugar utilization and bioethanol production profiles of S. cerevisiae CT2 pro from (**a**) 50 mM citrate buffer, (**b**) 5 mM citrate buffer, and (**c**) 0.5 mM citrate buffer hydrolysates.

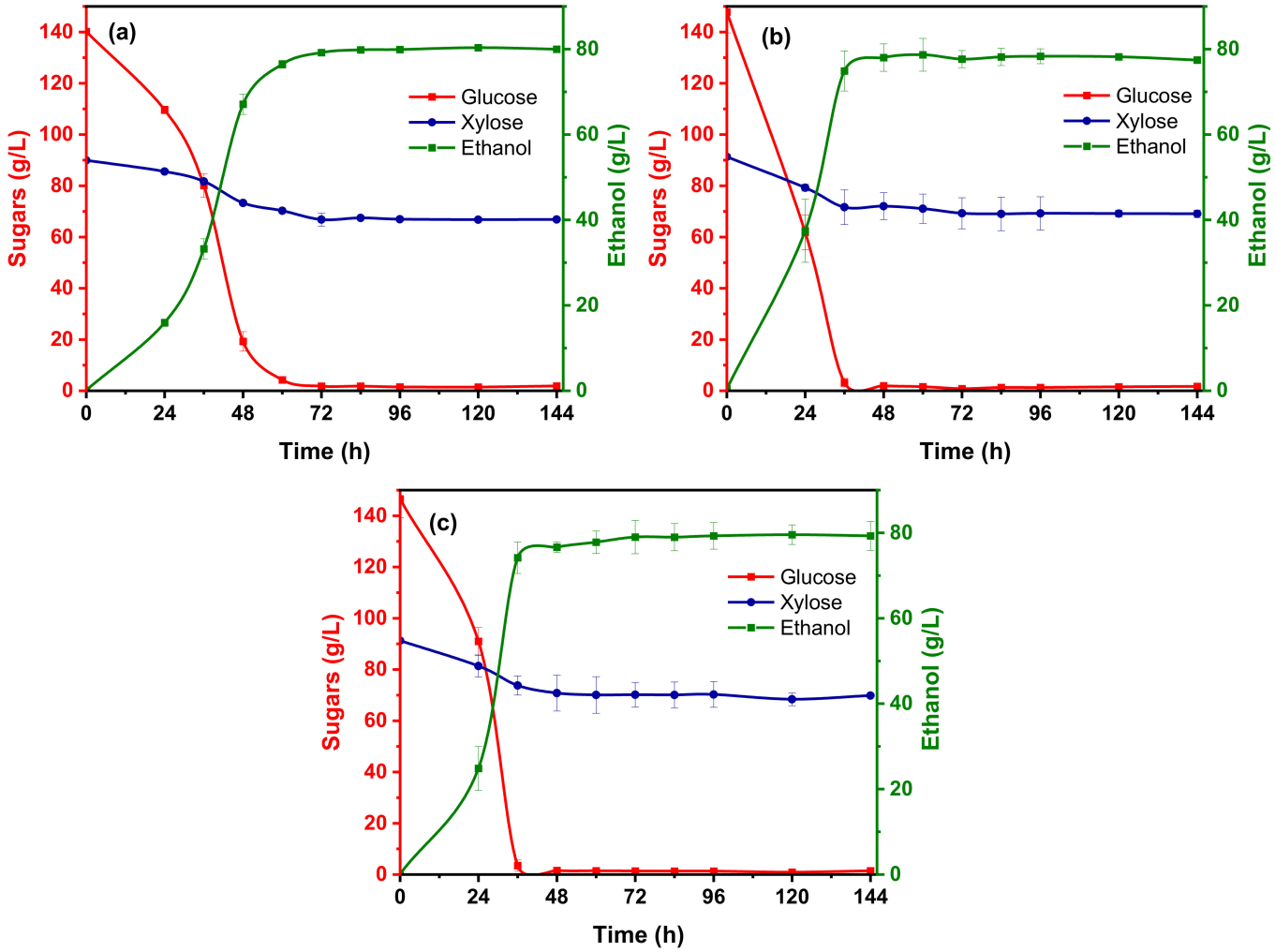

Figure 6. Sugar utilization and bioethanol production profile of co-culture (ethanol red and commercial recombinant *S. cerevisiae*) fermentation of different hydrolysates: (**a**) 50 mM citrate buffer, (**b**) 5 mM citrate buffer, and (**c**) 0.5 mM citrate buffer.

Glucose was rapidly fermented in all the yeast fermentations and was entirely or nearly exhausted within 36–48 h (Figures 4–6), except for the 50 mM citrate buffered co-culture fermentation, which took 60 h. Xylose was fermented much slower and much was left unfermented even after 144 h. It was co-metabolized with glucose. For most microbes, glucose is metabolized prior to xylose (termed catabolite repression). However, this is not the case for xylose-fermenting *Saccharomyces* strains because the inserted xylose metabolism genes are expressed using constitutive promoters. In fact, CT2 Pro primarily consumed xylose while glucose was still present in the culture. In contrast, the commercial xylose fermenting yeast strain consumed xylose at a steady rate throughout the fermentation. While glucose and xylose were co-utilized, literature suggests that high-glucose concentrations competitively inhibit xylose uptake through the xylose transporter [34].

The primary conclusion is that lowering the citrate buffer concentration led to a faster fermentation rate (Figure S3) and the same final ethanol yield. These results are in partial agreement with an earlier study using dilute-acid pretreated sorghum stalks that compared 0.5 mM with 50 mM citrate buffer for enzymatic hydrolysis and fermentation [19]. In this study, both productivity and yield were improved by reducing citrate from 50 mM to 0.5 mM. However, in their study following pretreatment, the solids were washed with water prior to treatment with cellulases such that the microbial inhibitors present after pretreatment would have been reduced. It might seem odd that citrate concentration would have a detrimental effect because it is routinely used to buffer *Saccharomyces* fermentations. However, hydrolysates contain numerous potential fermentation inhibitors which act in concert. Therefore, it could be that the interaction of excess citrate with other components in the hydrolysate are responsible for the partial inhibition of yeast growth and fermentation. Furthermore, in the beginning of the fermentation, *Saccharomyces* reduces furans to the more benign alcohol form [35]. Attenuation of the furans at the beginning of the fermentation might explain all the yeast cultures achieving similar maximum ethanol titers regardless of citrate concentration.

The second conclusion is that the fermentations were successful. Fermentations achieved 70.4–81.7 g/L ethanol, which is exceptional for cellulosic fermentation and far exceeds the 40 g/L threshold supposed for commercial interest that is based on distillation performance [36]. The average ethanol productivity on glucose, which was well in excess of 1.18 g/L/h, was also high relative to most other studies. However, only approximately 30–50% of provided xylose was fermented, which offers a clear opportunity for improvement. Xylose metabolism generates less ATP than glucose and this is believed to make the yeast more sensitive to inhibitors when growing on xylose, especially undissociated acetic acid [37]. For this same reason, the yeast could also be more sensitive to ethanol inhibition than when fermenting glucose. Further work is warranted to determine the underlying reason for stalled uptake of xylose in this system. There are only a handful of reports using lignocellulosic hydrolysates, all recent, where glucose and xylose are co-fermented to yield greater than 70 g/L ethanol [38–41]. For yeast, these studies applied simultaneous saccharification and co-fermentation of glucose and xylose (SSCF). SSCF are often adjusted to ensure a slow release of glucose, which promotes better xylose utilization [38]. Other strategies than SSCF that have or could be used to improve xylose fermentation are processing biomass to produce a xylose-enriched stream [42], adaptive laboratory evolution [43], cell recycle to prime enzymes for xylose utilization [44] and enhance xylose uptake [45], slow auxiliary glucose feed [46], or in situ removal of ethanol [47].

The current study's results are in agreement with the DMR (deacetylation followed by mechanical refining) process for bioethanol production from the lignocellulosic biomass [40]. In the DMR process, corn stover was deacetylated with sodium hydroxide and the generated black liquor was drained. Water was added to wash the solids partially and drained. Further, solids were dewatered using a screw press to attain 45 to 50% (*w*/*w*) insoluble solids. Dewatered solids were mechanically refined by disc milling (first-stage refining), followed by Szego milling (second-stage refining). Enzymatic hydrolysis of DMR solids yielded 230 g/L of monomeric sugars at 28 wt% solids loading using 20 mg of total pro-

tein per g of cellulose. In the subsequent fermentation of hydrolysates by recombinant *Zymomonas mobilis* 13-H-9-2, 81 to 86 g/L ethanol titers were attained with an ethanol yield of 81 to 90% (0.41 to 0.46 g_p/g_s). In this study, monomeric sugars yields (222 to 241 g/L), ethanol titers (81 g/L), and ethanol yields (0.48 g_p/g_s) were observed. The current process involves hydrothermal pretreatment, drying, disc milling, enzymatic hydrolysis, and fermentation. No acids or alkalis were used in the pretreatment process and washing of pretreated biomass is not required [20].

3.3.2. Microbial Lipid Production Cultures

R. toruloides was selected as biocatalyst for lipid production because of its strong ability to produce lipids and use a broad range of sugars [48]. The specific strain chosen was previously observed to be robust for growth on hydrolysates [14]. A pre-experiment was performed where the 0.5 mM and 50 mM hydrolysate diluted to either 75 g/L, 100 g/L, or 125 g/L total sugars to determine the optimal hydrolysate concentration. This experiment was conducted using a micro-culture plate format that allows for running 48 cultures simultaneously while conducting continuous monitoring of yeast biomass through the measurement of backscatter. Once the run was completed (96 h), each culture was sampled for the final lipid titer and optical densities.

The yeast grew at all three sugar concentrations buffered with either 0.5 mM or 50 mM citrate (Figure 7a). Using concentrated hydrolysate often leads to excess lag phases because of the presence of furans. Here, the lag phases lasted for similar times (approximately 18 h). There was also no discernable pattern in the final optical densities. However, this was not the case for final lipid values. Lipids varied from 6.22–11.89 g/L (Figure 7b). Lipids were higher or the same across sugar concentrations for the reduced citrate buffered cultures. Lipid concentrations were also higher or similar for the 100 versus 125 g/L sugar concentrations. Glucose was completely consumed by all the yeast cultures. Xylose was completely consumed for the 75 g/L and 100 g/L cultures. For the 125 g/L sugar concentration, residual xyloses were 1.0 g/L and 0.24 g/L for the 50 mM and 0.5 mM buffered cultures, respectively. The pHs showed an interesting trend, they rose from 6.0 to 7.9–8.2. Most microbial cultures become more acidic because of the production of carbonic acid. In summary, the yeast grew on hydrolysate up to a concentration of 125 g/L sugars and had the maxima lipid yields at 100 g/L sugars.

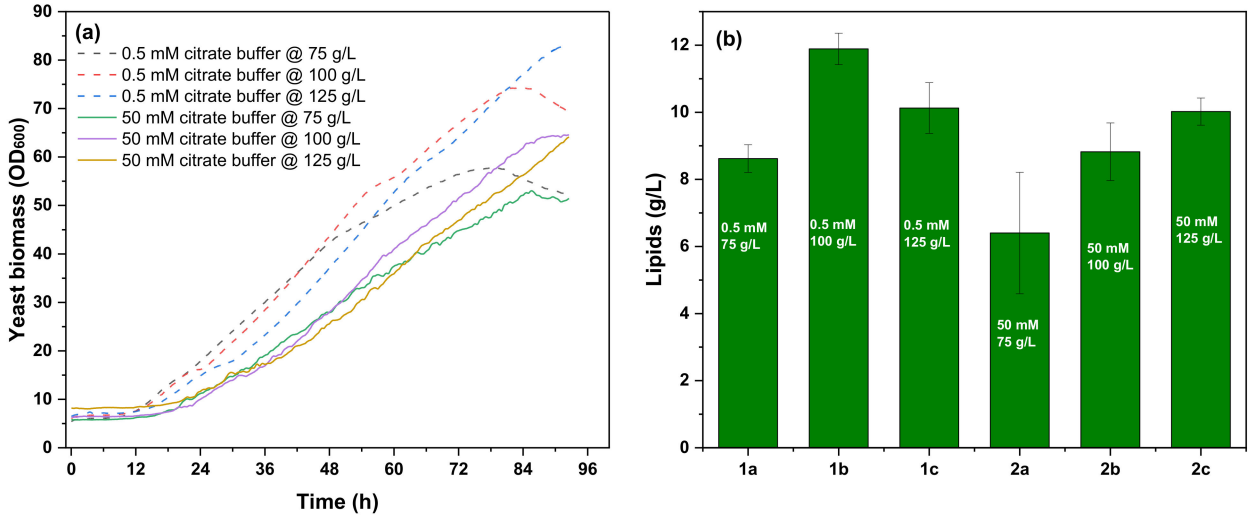

Figure 7. Biolector studies for optimization of sugar concentration: (**a**) microbial growth profile; (**b**) lipid titers from 50 mM and 0.5 mM citrate buffer strengths.

Oleaginous yeast are typically grown in nitrogen-limited medium. The yeast grow until the nitrogen is exhausted and in response to nitrogen starvation convert the remaining sugars to lipids, which they store internally in the form of droplets [49]. They continue to

accumulate lipids as long as there are remaining sugars after which the lipid titer declines as they begin to deplete their stored lipids. For this reason, the 100 g/L sugar hydrolysates were repeated in flask cultures, which afforded periodic sampling.

Yeast completely consumed sugars (glucose and xylose) during the fermentation of 50 mM and 0.5 mM citrate buffer hydrolysates at 77.5 h and produced 13.2 g/L lipids, which was similar for both hydrolysates (Figure 8). At 96 h fermentation time, lipid concentration decreased to 10.2 g/L. The lower lipid titer was due to the exhaustion of sugars and the use of lipids as a carbon source by the microorganisms.

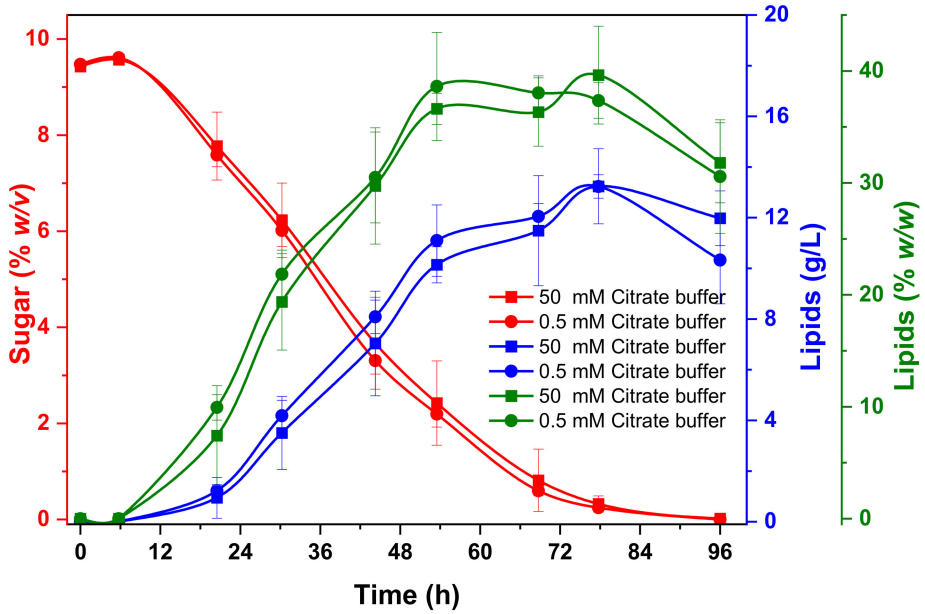

Figure 8. Lipid production studies from 50 mM and 0.5 mM citrate buffer strengths at 100 g/L sugar concentration: sugar utilization, lipid production, and lipid accumulation profiles.

Xylose consumption was repressed in the presence of glucose (Figure S4a,b). Glucose and xylose were depleted within 44.5 h and 77.5 h, respectively. These were followed by L-arabinose, which was depleted by 96 h. In addition to glucose, xylose, and arabinose, *R. toruloides* Y-6987 also consumed acetic acid early on in the culture (5.75 h), which might have contributed to the increase in pHs observed for the cultures (Figure S4c). Formation of arabitol was observed at 44.25 h, but no arabitol was detected at 68.75 h. Biomass growth (dry weight basis), lipid titer, and increment of pH profile were observed during the fermentation of both the 50 mM and 0.5 mM hydrolysates (Figures 7 and S4c). In addition, yeast lipid content was calculated using the following equation (Equation (16)). As expected, lipid accumulations were similar (38.5% w/w) for the 50 mM and 0.5 mM citrate buffer cultures (Figure 8). Sugar consumption rate, lipid productivity, and percentage of lipid accumulation were slightly higher during the fermentation of 0.5 mM citrate buffer hydrolysate compared with 50 mM citrate buffer hydrolysate (Figures 8 and S4a–c).

$$Lipid\ accumulation\ (\%) = \left[\frac{Lipid\ titer\ (g/L)}{Cell\ dry\ weight\ (g/L)}\right] \times 100 \quad (14)$$

Therefore, reduced citrated concentration did not affect lipid production or end titer. Lipid titers were similar to that reported earlier by this laboratory (11.9 g/L vs. 13.2 g/L here, [14]). This yield is also similar to others observed for hydrolysates in batch cultures for *R. toruloides* (1.23–13.8 g/L, [50]). However, dramatically improved lipid titers have been reported for herbaceous hydrolysates using fed-batch (32 g/L) or two-stage (19.09 g/L and 26.2 g/L) processes for this yeast species [14,50,51].

4. Conclusions

In this study, more than 70% and 79% glucose and xylose yields are achieved at 50% (*w*/*v*) solids loading in all the citrate buffer strengths (50 mM, 5 mM, and 0.5 mM). This eventually indicates that citrate buffer strength was significantly reduced to 100-fold for the enzymatic hydrolysis of hydrothermal pretreated biomass. Moreover, conducting the enzymatic hydrolysis of hydrothermal pretreated biomass in low citrate buffer was beneficial to the subsequent fermentation of the hydrolysate to achieve an industrial titer of bioethanol within 48 h of incubation. Compared with the hydrolysate prepared with 5 mM and 0.5 mM citrate buffer strengths, sugar consumption rate and ethanol productivities were comparatively higher for yeasts fermenting hydrolysate prepared with the 50 mM citrate buffer strength during enzyme hydrolysis. Even though ethanol yield did not vary with buffer concentration, commercial recombinant *S. cerevisiae*, and co-culture of (Ethanol Red plus commercial recombinant *S. cerevisiae*), produced slightly higher bioethanol titers (80.3–81.7 g/L) than *S. cerevisiae* CT2 pro (70.4–71.3 g/L). Moreover, lipid yield (13.2 g/L) and its accumulation (38.5% *w*/*w*) in the *R. toruloides* Y-6987 were also found to be similar during the fermentation of 50 mM and 0.5 mM citrate buffer hydrolysates at 100 g/L sugar concentration. Therefore, based on the sugars yielded during the enzymatic hydrolysis, ethanol productivity and yield during fermentation and lipid yield by an oleaginous yeast, it can be concluded that conducting the enzymatic hydrolysis of hydrothermal pretreated lignocellulosic biomass at low citrate buffer strength is beneficial. The current process has overcome the dilute acid or alkali pretreatment step and subsequent washing of pretreated solids barriers to achieve above 81 g/L bioethanol from lignocellulosic biomass.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9010005/s1, Table S1: ANOVA results for comparing the enzymatic hydrolysis results from three citrate buffers (50, 5, 0.5 mM). Figure S1: Sugar (glucose plus xylose) yield from enzymatic hydrolysis of hydrothermal pretreated bioenergy sorghum biomass at 20% to 60% (*w*/*v*) solid loadings in different citrate buffer strengths. Figure S2: Sugar (glucose plus xylose) yield at 30% to 50% (*w*/*v*) solid loadings of enzymatic hydrolysis of hydrothermally pretreated bioenergy sorghum biomass in the scale-up study at different citrate buffer strengths. Figure S3: Ethanol productivity profile of (a) commercial recombinant S. cerevisiae (b) S. cerevisiae CT2 Pro and (c) Co-culture (ethanol red and commercial recombinant S. cerevisiae) on different hydrolysates 50, 5, and mM citrate buffer. Figure S4: Glucose and xylose utilization profiles of R. toruloides Y-6987 during the fermentation of enzymatic hydrolysates derived from (a) 50 mM citrate buffer strength (b) 0.5 mM citrate buffer strength at 100 g/L sugar concentration and (c) pH and cell dry weight.

Author Contributions: N.N.D.: conceptualization, methodology, investigation, experiments, data analysis, and original draft preparation. P.J.S.: preparation of protocols for the lipid of experiments. B.S.D.: supervision, data analysis, results validation, and corrected original draft. Y.-S.J.: data analysis, results validation, and corrected original draft. V.S.: supervision, results validation, corrected original draft, and reviewing and editing of the finalized manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Bruce Dien and Patricia Slininger received financial support from the United States Department of Agriculture, Agricultural Research Service (CRIS Number 5010-41000-189-00D). Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer. Authors are thankful to the DOE Center for Advanced Bioenergy and Bioproducts Innovation (U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Number DE-SC0018420) for funding this research work. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Energy.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Acknowledgments: The authors would like to thank August Schetter for harvesting feedstock, Novozymes North America for providing enzymes, Stephanie Thompson for assisting with lipid yeast study, Eric Wolf and IBRL (Integrated Bioprocessing Research Laboratory, UIUC) staff for assisting with pilot-scale pretreatment, and Kristen K. Eilts for assisting with HPLC analysis.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Keasling, J.; Garcia Martin, H.; Lee, T.S.; Mukhopadhyay, A.; Singer, S.W.; Sundstrom, E. Microbial Production of Advanced Biofuels. *Nat. Rev. Microbiol.* **2021**, *19*, 701–715. [CrossRef] [PubMed]
2. Field, J.L.; Richard, T.L.; Smithwick, E.A.H.; Cai, H.; Laser, M.S.; LeBauer, D.S.; Long, S.P.; Paustian, K.; Qin, Z.; Sheehan, J.J.; et al. Robust Paths to Net Greenhouse Gas Mitigation and Negative Emissions via Advanced Biofuels. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 21968–21977. [CrossRef] [PubMed]
3. Vohra, M.; Manwar, J.; Manmode, R.; Padgilwar, S.; Patil, S. Bioethanol Production: Feedstock and Current Technologies. *J. Environ. Chem. Eng.* **2014**, *2*, 573–584. [CrossRef]
4. Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y.Y.; Holtzapple, M.; Ladisch, M. Features of Promising Technologies for Pretreatment of Lignocellulosic Biomass. *Bioresour. Technol.* **2005**, *96*, 673–686. [CrossRef]
5. Kim, S.M.; Dien, B.S.; Singh, V. Promise of Combined Hydrothermal/Chemical and Mechanical Refining for Pretreatment of Woody and Herbaceous Biomass. *Biotechnol. Biofuels* **2016**, *9*, 97. [CrossRef] [PubMed]
6. Cheng, M.H.; Dien, B.S.; Lee, D.K.; Singh, V. Sugar Production from Bioenergy Sorghum by Using Pilot Scale Continuous Hydrothermal Pretreatment Combined with Disk Refining. *Bioresour. Technol.* **2019**, *289*, 121663. [CrossRef]
7. Cheng, M.H.; Kadhum, H.J.; Murthy, G.S.; Dien, B.S.; Singh, V. High Solids Loading Biorefinery for the Production of Cellulosic Sugars from Bioenergy Sorghum. *Bioresour. Technol.* **2020**, *318*, 124051. [CrossRef]
8. Deshavath, N.N.; Mogili, N.V.; Dutta, M.; Goswami, L.; Kushwaha, A.; Veeranki, V.D.; Goud, V.V. Role of Lignocellulosic Bioethanol in the Transportation Sector: Limitations and Advancements in Bioethanol Production from Lignocellulosic Biomass. In *Waste-to-Energy Approaches Towards Zero Waste*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 57–85. ISBN 9780323853873.
9. Wan, G.; Zhang, Q.; Li, M.; Jia, Z.; Guo, C.; Luo, B.; Wang, S.; Min, D. How Pseudo-Lignin Is Generated during Dilute Sulfuric Acid Pretreatment. *J. Agric. Food Chem.* **2019**, *67*, 10116–10125. [CrossRef]
10. Qin, L.; Li, W.C.; Liu, L.; Zhu, J.Q.; Li, X.; Li, B.Z.; Yuan, Y.J. Inhibition of Lignin-Derived Phenolic Compounds to Cellulase. *Biotechnol. Biofuels* **2016**, *9*, 70. [CrossRef]
11. Pisithkul, T.; Jacobson, T.B.; O'Brien, T.J.; Stevenson, D.M.; Amador-Noguez, D. Phenolic Amides Are Potent Inhibitors of de Novo Nucleotide Biosynthesis. *Appl. Environ. Microbiol.* **2015**, *81*, 5761–5772. [CrossRef]
12. Deshavath, N.N.; Veeranki, V.D.; Goud, V.V. Lignocellulosic Feedstocks for the Production of Bioethanol: Availability, Structure, and Composition. In *Sustainable Bioenergy*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 1–19. ISBN 9780128176542.
13. Tarabanko, N.; Baryshnikov, S.V.; Kazachenko, A.S.; Miroshnikova, A.V.; Skripnikov, A.M.; Lavrenov, A.V.; Taran, O.P.; Kuznetsov, B.N. Hydrothermal Hydrolysis of Microcrystalline Cellulose from Birch Wood Catalyzed by Al2O3-B2O3 Mixed Oxides. *Wood Sci. Technol.* **2022**, *56*, 437–457. [CrossRef]
14. Cheng, M.H.; Dien, B.S.; Jin, Y.S.; Thompson, S.; Shin, J.; Watson Slininger, P.J.; Qureshi, N.; Singh, V. Conversion of High-Solids Hydrothermally Pretreated Bioenergy Sorghum to Lipids and Ethanol Using Yeast Cultures. *ACS Sustain. Chem. Eng.* **2021**, *9*, 8515–8525. [CrossRef]
15. Jin, M.; Slininger, P.J.; Dien, B.S.; Waghmode, S.; Moser, B.R.; Orjuela, A.; da Costa Sousa, L.; Balan, V. Microbial Lipid-Based Lignocellulosic Biorefinery: Feasibility and Challenges. *Trends Biotechnol.* **2015**, *33*, 43–54. [CrossRef] [PubMed]
16. Kwak, S.; Jin, Y.S. Production of Fuels and Chemicals from Xylose by Engineered Saccharomyces Cerevisiae: A Review and Perspective. *Microb. Cell Fact.* **2017**, *16*, 82. [CrossRef]
17. Tsai, C.S.; Kong, I.I.; Lesmana, A.; Million, G.; Zhang, G.C.; Kim, S.R.; Jin, Y.S. Rapid and Marker-Free Refactoring of Xylose-Fermenting Yeast Strains with Cas9/CRISPR. *Biotechnol. Bioeng.* **2015**, *112*, 2406–2411. [CrossRef]
18. Deshavath, N.N.; Goud, V.V.; Veeranki, V.D. Liquefaction of Lignocellulosic Biomass through Biochemical Conversion Pathway: A Strategic Approach to Achieve an Industrial Titer of Bioethanol. *Fuel* **2021**, *287*, 119545. [CrossRef]
19. Deshavath, N.N.; Goud, V.V.; Veeranki, V.D. Curtailing Citrate Buffer Inhibition Effect on S. Cerevisiae to Enhance the Fermentability of Cellulosic Hydrolysate. *J. Environ. Chem. Eng.* **2021**, *9*, 105696. [CrossRef]
20. Tao, L.; Schell, D.; Davis, R.; Tan, E.; Elander, R.; Bratis, A. *NREL 2012 Achievement of Ethanol Cost Targets: Biochemical Ethanol Fermentation via Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2014.
21. Arora, A.; Carrier, D.J. Understanding the Pine Dilute Acid Pretreatment System for Enhanced Enzymatic Hydrolysis. *ACS Sustain. Chem. Eng.* **2015**, *3*, 2423–2428. [CrossRef]
22. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Nrel, D.C. *Determination of Structural Carbohydrates and Lignin in Biomass Determination of Structural Carbohydrates and Lignin in Biomass*; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2012.

23. Deshavath, N.N.; Dasu, V.V.; Goud, V.V.; Rao, P.S. Development of Dilute Sulfuric Acid Pretreatment Method for the Enhancement of Xylose Fermentability. *Biocatal. Agric. Biotechnol.* **2017**, *11*, 224–230. [CrossRef]
24. Dien, B.S.; Slininger, P.J.; Kurtzman, C.P.; Moser, B.R.; O'Bryan, P.J. Identification of Superior Lipid Producing Lipomyces and Myxozyma Yeasts. *AIMS Environ. Sci.* **2016**, *3*, 1–20. [CrossRef]
25. Rodríguez-Zúñiga, U.F.; Cannella, D.; Giordano, R.D.C.; Giordano, R.D.L.C.; Jørgensen, H.; Felby, C. Lignocellulose Pretreatment Technologies Affect the Level of Enzymatic Cellulose Oxidation by LPMO. *Green Chem.* **2015**, *17*, 2896–2903. [CrossRef]
26. Sun, D.; Lv, Z.W.; Rao, J.; Tian, R.; Sun, S.N.; Peng, F. Effects of Hydrothermal Pretreatment on the Dissolution and Structural Evolution of Hemicelluloses and Lignin: A Review. *Carbohydr. Polym.* **2022**, *281*, 119050. [CrossRef] [PubMed]
27. Sanchez-Silva, L.; López-González, D.; Villaseñor, J.; Sánchez, P.; Valverde, J.L. Thermogravimetric-Mass Spectrometric Analysis of Lignocellulosic and Marine Biomass Pyrolysis. *Bioresour. Technol.* **2012**, *109*, 163–172. [CrossRef] [PubMed]
28. Deshavath, N.N.; Mahanta, S.; Goud, V.V.; Dasu, V.V. Chemical Composition Analysis of Various Genetically Modified Sorghum Traits: Pretreatment Process Optimization and Bioethanol Production from Hemicellulosic Hydrolyzates without Detoxification. *J. Environ. Chem. Eng.* **2018**, *6*, 5625–5634. [CrossRef]
29. Jing, X.; Zhang, X.; Bao, J. Inhibition Performance of Lignocellulose Degradation Products on Industrial Cellulase Enzymes during Cellulose Hydrolysis. *Appl. Biochem. Biotechnol.* **2009**, *159*, 696–707. [CrossRef]
30. Zhang, J.; Chu, D.; Huang, J.; Yu, Z.; Dai, G.; Bao, J. Simultaneous Saccharification and Ethanol Fermentation at High Corn Stover Solids Loading in a Helical Stirring Bioreactor. *Biotechnol. Bioeng.* **2010**, *105*, 718–728. [CrossRef]
31. Jin, M.; Gunawan, C.; Uppugundla, N.; Balan, V.; Dale, B.E. A Novel Integrated Biological Process for Cellulosic Ethanol Production Featuring High Ethanol Productivity, Enzyme Recycling and Yeast Cells Reuse. *Energy Environ. Sci.* **2012**, *5*, 7168–7175. [CrossRef]
32. Humbird, D.; Davis, R.; Tao, L.; Kinchin, C.; Hsu, D.; Aden, A.; Schoen, P.; Lukas, J.; Olthof, B.; Worley, M.J.B.B.; et al. *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*; National Renewable Energy Lab. (NREL): Golden, CO, USA, 2011.
33. Casey, E.; Mosier, N.S.; Adamec, J.; Stockdale, Z.; Ho, N.; Sedlak, M. Effect of Salts on the Co-Fermentation of Glucose and Xylose by a Genetically Engineered Strain of Saccharomyces Cerevisiae. *Biotechnol. Biofuels* **2013**, *6*, 83. [CrossRef]
34. Lee, W.J.; Kim, M.D.; Ryu, Y.W.; Bisson, L.; Seo, J.H. Kinetic Studies on Glucose and Xylose Transport in Saccharomyces Cerevisiae. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 186–191. [CrossRef]
35. Liu, Z.L.; Slininger, P.J.; Dien, B.S.; Berhow, M.A.; Kurtzman, C.P.; Gorsich, S.W. Adaptive Response of Yeasts to Furfural and 5-Hydroxymethylfurfural and New Chemical Evidence for HMF Conversion to 2,5-Bis-Hydroxymethylfuran. *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 345–352. [CrossRef]
36. Lau, M.W.; Dale, B.E. Cellulosic Ethanol Production from AFEX-Treated Corn Stover Using Saccharomyces Cerevisiae 424A(LNH-ST). *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1368–1373. [CrossRef] [PubMed]
37. Casey, E.; Sedlak, M.; Ho, N.W.Y.; Mosier, N.S. Effect of Acetic Acid and PH on the Cofermentation of Glucose and Xylose to Ethanol by a Genetically Engineered Strain of Saccharomyces Cerevisiae. *FEMS Yeast Res.* **2010**, *10*, 385–393. [CrossRef] [PubMed]
38. Yuan, X.; Shen, G.; Chen, S.; Chen, X.; Zhang, C.; Liu, S.; Jin, M. Modified Simultaneous Saccharification and Co-Fermentation of DLC Pretreated Corn Stover for High-Titer Cellulosic Ethanol Production without Water Washing or Detoxifying Pretreated Biomass. *Energy* **2022**, *247*, 123488. [CrossRef]
39. Shen, G.; Yuan, X.; Chen, S.; Liu, S.; Jin, M. High Titer Cellulosic Ethanol Production from Sugarcane Bagasse via DLCA Pretreatment and Process Development without Washing/Detoxifying Pretreated Biomass. *Renew. Energy* **2022**, *186*, 904–913. [CrossRef]
40. Chen, X.; Kuhn, E.; Jennings, E.W.; Nelson, R.; Tao, L.; Zhang, M.; Tucker, M.P. DMR (Deacetylation and Mechanical Refining) Processing of Corn Stover Achieves High Monomeric Sugar Concentrations (230 g L^{-1}) during Enzymatic Hydrolysis and High Ethanol Concentrations (>10% *v*/*v*) during Fermentation without Hydrolysate Purification or concentration. *Energy Environ. Sci.* **2016**, *9*, 1237–1245. [CrossRef]
41. Liu, G.; Zhang, Q.; Li, H.; Qureshi, A.S.; Zhang, J.; Bao, X.; Bao, J. Dry Biorefining Maximizes the Potentials of Simultaneous Saccharification and Co-Fermentation for Cellulosic Ethanol Production. *Biotechnol. Bioeng.* **2018**, *115*, 60–69. [CrossRef]
42. Cheng, M.H.; Sun, L.; Jin, Y.S.; Dien, B.; Singh, V. Production of Xylose Enriched Hydrolysate from Bioenergy Sorghum and Its Conversion to β-Carotene Using an Engineered Saccharomyces Cerevisiae. *Bioresour. Technol.* **2020**, *308*, 123275. [CrossRef]
43. Ko, J.K.; Enkh-Amgalan, T.; Gong, G.; Um, Y.; Lee, S.M. Improved Bioconversion of Lignocellulosic Biomass by Saccharomyces Cerevisiae Engineered for Tolerance to Acetic Acid. *GCB Bioenergy* **2020**, *12*, 90–100. [CrossRef]
44. Slininger, P.J.; Thompson, S.R.; Weber, S.; Liu, Z.L.; Moon, J. Repression of Xylose-Specific Enzymes by Ethanol in Scheffersomyces (Pichia) Stipitis and Utility of Repitching Xylose-Grown Populations to Eliminate Diauxic Lag. *Biotechnol. Bioeng.* **2011**, *108*, 1801–1815. [CrossRef]
45. Roca, C.; Olsson, L. Increasing Ethanol Productivity during Xylose Fermentation by Cell Recycling of Recombinant Saccharomyces Cerevisiae. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 560–563. [CrossRef]
46. Olofsson, K.; Wiman, M.; Lidén, G. Controlled Feeding of Cellulases Improves Conversion of Xylose in Simultaneous Saccharification and Co-Fermentation for Bioethanol Production. *J. Biotechnol.* **2010**, *145*, 168–175. [CrossRef] [PubMed]
47. Kumar, D.; Juneja, A.; Singh, V. Fermentation Technology to Improve Productivity in Dry Grind Corn Process for Bioethanol Production. *Fuel Process. Technol.* **2018**, *173*, 66–74. [CrossRef]

48. Xu, J.; Liu, D. Exploitation of Genus Rhodosporidium for Microbial Lipid Production. *World J. Microbiol. Biotechnol.* **2017**, *33*, 54. [CrossRef] [PubMed]
49. Ageitos, J.M.; Vallejo, J.A.; Veiga-Crespo, P.; Villa, T.G. Oily Yeasts as Oleaginous Cell Factories. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1219–1227. [CrossRef] [PubMed]
50. Slininger, P.J.; Dien, B.S.; Kurtzman, C.P.; Moser, B.R.; Bakota, E.L.; Thompson, S.R.; O'Bryan, P.J.; Cotta, M.A.; Balan, V.; Jin, M.; et al. Comparative Lipid Production by Oleaginous Yeasts in Hydrolyzates of Lignocellulosic Biomass and Process Strategy for High Titers. *Biotechnol. Bioeng.* **2016**, *113*, 1676–1690. [CrossRef] [PubMed]
51. Fei, Q.; O'Brien, M.; Nelson, R.; Chen, X.; Lowell, A.; Dowe, N. Enhanced Lipid Production by Rhodosporidium Toruloides Using Different Fed-Batch Feeding Strategies with Lignocellulosic Hydrolysate as the Sole Carbon Source. *Biotechnol. Biofuels* **2016**, *9*, 130. [CrossRef] [PubMed]