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Effectiveness of Tannin Removal by Alkaline Pretreatment on Sorghum Ethanol Production

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Abstract: Sorghum has been proposed as a complement or replacement for corn in ethanol production. One difference between sorghum and corn is the presence of tannins, which may affect enzymatic activity. High-tannin sorghum hybrid XM217 was used to analyze the effect of tannin removal by the alkaline pretreatment of sorghum for ethanol production. A laboratory-scale dry-milling process was used on treated sorghum/corn blends to generate mash that was fermented by *Saccharomyces cerevisiae* and then compared to a 100% untreated sorghum control. Cellulase was added to a similar set of mash to determine the feasibility of the tannin-removal treatment as a pretreatment method for cellulosic ethanol production. Theoretical ethanol yield increased from 68.2 ± 1.5% to 78.5 ± 2.5% for alkaline-pretreated sorghum vs. untreated sorghum, with a corresponding increase in mean ethanol concentrations from 8.02 ± 0.15 to 9.39 ± 0.26% *w/v*. The average theoretical ethanol yield increased from 69.8 ± 1.7% to 94.6 ± 1.9% when using cellulase with untreated and treated sorghum. The use of alkaline tannin removal resulted in a significant increase in the theoretical ethanol yield obtained when using 100% sorghum, when compared to the theoretical ethanol yield obtained when using 100% corn. The combination of cellulase and alkaline tannin removal improved the yield of ethanol in all cases compared to the experiments without cellulase.

Keywords: ethanol; tannins; sorghum; biofuels

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1. Introduction

Bioethanol is a renewable fuel source that allows us to reduce our dependence on fossil fuels. However, the sustainability of bioethanol produced from corn feedstock has been questioned due to potential soil erosion, the loss of biodiversity, high use of nitrogen fertilizer, and significant use of land and water [1–3]. In the US, 30% of the corn harvested is used to produce bioethanol [4]. This represents 95% of the raw material used in this process, with the remaining material coming from wheat, barley, cheese whey, and beverage residues [5].

In order to improve the sustainability of bioethanol production, sorghum has been proposed as a complement or replacement for corn [6]. The use of sorghum has several advantages for ethanol production. First, sorghum has a composition that is similar to corn. An analysis by the US Grain Council of corn and sorghum samples indicated that protein (8.6 vs. 8.5%, dwb), starch (72.5 vs. 72.6%), and oil (4.0% vs. 4.4%) contents, respectively, varied little within the two grains [7]. This similar composition allows sorghum to be used interchangeably with corn in a dry-grind corn ethanol plant with minimal engineering modifications. Second, sorghum is well-adapted to environments with high temperatures and is tolerant to drought stress [8,9]; therefore, sorghum cultivation can be extended to dry areas [6,10,11] with reduced water consumption.

One of the differences between corn and sorghum is the presence of tannins in sorghum. Tannins are compounds derived from tannic (phenolic) acid. Red and white sorghum

contain different quantities of condensed tannins. Tannins are located in the testa layer of grain and increase resistance to insects and weather conditions [6], thus acting as a defense mechanism for the plant. Tannins also are known to bind to proteins, which may interfere with the activity of enzymes used in bioethanol production to hydrolyze starch and dextrins to simple sugars. Tannins also increase the viscosity of the mixture [12,13], which may pose a problem in a biorefinery because of agitation and pumping issues.

The objective of this research was to investigate the impact of tannin removal by the alkaline pretreatment of sorghum for ethanol production. The goal was to find the combination of corn and treated sorghum that produced the highest ethanol yield. Further, the effect of cellulase addition using a commercial cellulase after tannin removal on ethanol production was evaluated.

2. Materials and Methods

2.1. Materials

No. 2 Yellow Corn, which was grown in Anderson County, SC, was obtained from a local provider (Griff's Farm & Home Center, Pendleton, SC, Portland, OR, USA). High-tannin sorghum hybrid XM217, harvested near Lubbock, TX, was provided by Sorghum Partners Inc. Both grains were initially placed in a freezer for 7 days to kill any live insects, and then stored in plastic bags at room temperature and with low ambient humidity. The corn and the treated and untreated sorghum were milled separately in a coffee grinder. The ground product was sieved with a 2 mm screen (mesh 10). The passing grain was stored at 0 °C. Moisture content was determined by drying 2 g of the sample at 103 °C +/− 2 °C for 8 h until a constant weight was achieved.

Liquozyme SC DS® (α-amylase), Spirizyme Ultra® (glucoamylase), and CTec2® (cellulase), provided by Novozymes (Franklinton, NC, USA), and active dry yeast C6 FUEL™ (*Saccharomyces cerevisiae*), provided by Lallemand Biofuels & Distilled Spirits (Duluth, GA), were stored at 4 °C. Liquozyme SC DS® and Spirizyme Ultra® were diluted to 1:50 (*v/v*) with deionized water and stored at 4 °C. Active dry yeast was rehydrated by mixing 2.5 g of yeast with 50 mL of deionized water and stirring for 30 min. This solution was prepared immediately prior to the addition to fermentation flasks.

A 10% *w/v* solution of urea in deionized water was made and kept refrigerated. All chemicals were purchased from VWR (Atlanta, GA, USA) and were analytical grade. Flasks (250 mL) and stoppers used for fermentations were steam-sterilized at 121 °C for 20 min.

2.2. Methods

2.2.1. Tannin Removal Procedure

The tannin removal procedure for sorghum was modified from Armstrong et al. [14]. Samples were presoaked in deionized water at 60 °C for five minutes with constant stirring. After draining, 0.80 kg of sorghum was soaked in 1 L of 20% sodium hydroxide at 70 °C for eight minutes. The grain was drained in a metallic screen and rinsed with 50 °C tap water until pH 8 was reached. The samples were dried in a forced-air oven at 70 °C for 6 h and then stored. The original procedure included a final neutralization step with acetic acid, which was not used in this study to avoid the harmful effects of residual acetic acid on fermentation.

2.2.2. Mash Liquefaction

Mixtures of 400 g of treated sorghum/corn were prepared as 100/0, 75/25, 50/50, 25/75, and 0/100% (*w/w*). An additional 400 g of untreated sorghum was also prepared. The grains were placed in a 3 L stainless steel beaker and 1200 g of water was added, yielding slurries with 25% solids. The pH of each was adjusted to 5.5 with 10 N sulfuric acid.

The beakers were placed in a water bath to maintain the temperature at 85 °C, and the slurries were agitated with a mechanical agitator at 500 rpm for 2 h. Liquozyme SC DS® was added as 0.058 g/100 g of dry solids (9.22 mL of diluted solution per batch). Deionized water was added to replace evaporative loss and maintain a total slurry weight of 1600 g.

After 2 h, the beaker was cooled to 50 °C in an ice bath. The liquefied slurry was split into two batches of 800 g of mash each.

2.2.3. Fermentations with No Cellulase Added

One beaker containing 800 g of liquefied mash was cooled to 40 °C in an ice bath. The mash was stirred at 300 rpm, and pH was adjusted to 3.90–4.10 with 10 N sulfuric acid. Spirizyme Ultra® (glucoamylase) was added as 0.116 g of enzyme per 100 g of dry solids (10.22 mL of diluted solution per 800 g batch) together with 3.20 mL of 10% urea solution. Agitation of the mash continued for 20 min to facilitate the dissolution of urea and even distribution of glucoamylase.

Mash was dispensed into 250 mL sterilized flasks at 100 g per flask, and 0.50 mL of the yeast slurry was added. Flasks were capped with #6 rubber stoppers. Six replicate flasks were incubated per treatment. An 18-gauge hypodermic needle was inserted into each stopper to allow pressure relief from carbon dioxide that formed during fermentation.

The flasks were incubated in an orbital shaker at 180 rpm and 32 °C for 96 h. Fermentation was considered to be completed at 96 h after reaching less than 5% of weight loss in eight hours. Each experiment had six replicates of each treatment.

Final samples were obtained for each flask and centrifuged at 4400 rpm for 15 min. Supernatants were filtered through a 0.45-micron syringe filter and stored for HPLC analysis.

2.2.4. Fermentations with Added Cellulase

The second beaker of 800 g of liquified mash was kept in a water bath at 50 °C with an agitation of 300 rpm, and 6 mL of CTec2® was added to the beaker and agitation continued for 1 h. The remaining processes were identical to those described above.

2.3. Analytical Methods

2.3.1. Tannin Analysis

The tannin content of sorghum was determined by using the acid–butanol assay modified from Porter et al. (1985) as described in Top et al. [15]. A 50 mg sorghum sample was placed into glass tubes, and 6 mL of butanol:HCl (95:5 *v/v*) reagent was added. Samples were incubated in a water bath at 90–95 °C for an hour, vortexing before and halfway through, and then cooled on ice.

The amount of anthocyanidin in the samples was quantified by measuring Abs_{550} (Jasco V-550 UV/VIS spectrophotometer, Jasco, Analytical Instruments, Easton, MD, USA) with comparison to a standard curve derived from cyanidin. The results are expressed as "cyanidin equivalents".

2.3.2. Starch Determination

An enzymatic method was used for starch determination. First, 100 mg of sample was weighed into glass test tubes, followed by the addition of 0.2 mL of 80% *v/v* aqueous ethanol. The contents were thoroughly mixed with a vortex mixer, and 3 mL of Liquozyme SC DS® was added to each tube. The tubes were placed in a boiling water bath for 6 min with intermediate vortexing, and then were transferred to a water bath at 50 °C, and 4 mL of 0.2 M sodium acetate buffer at pH 4.5 was added, followed by Spirizyme Ultra® (0.1 mL, 20 U). The tubes were stirred on a vortex mixer and incubated at 50 °C for 30 min. The entire contents of the test tubes were then transferred to 100 mL volumetric flasks, the volume was adjusted with distilled water, and it was mixed. A volume of ~1 mL was removed from each flask and centrifuged at 3000 rpm for 10 min on a microcentrifuge. Duplicates of 0.1 mL of the supernatant were transferred to test tubes, and 3 mL of GOPOD reagent was added to each tube. Then, the tubes were incubated at 50 °C for 20 min. Glucose controls and blanks were also prepared. Glucose controls consisted of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD reagent. A blank solution consisted of 0.1 mL of water and 3.0 mL of GOPOD reagent. Absorbance at 510 nm was determined for each sample, and the glucose control was set against the reagent blank.

2.3.3. Ethanol Determination

Ethanol concentrations at the end of fermentation were determined using a Shimadzu HPLC (Aminex® HPX-87H 300 × 7.8 mm ion-exclusion column (Bio-Rad Laboratories, CA, USA) and a RID-10A refractive index detector (Shimadzu)) with 0.005 M sulfuric acid mobile phase, operated at 60 °C with a flow of 0.60 mL/min using LCsolution version 1.25 software (Shimadzu Corporation, Kyoto, Japan). Two injections per sample were averaged and compared to the ethanol standard curve. The theoretical yield values for ethanol were calculated as shown in Appendix A.

2.3.4. Carbon Dioxide Determination

The mass of CO_2 produced per flask was estimated as the weight loss from the flask during fermentation. An 18-gauge syringe needle was placed in the rubber stopper of the fermentation flask to allow the release of CO_2 gas. The flasks were weighed six times during fermentation. Weight loss was used to assess the progress of ethanol production and as an estimate of the amount of CO_2 produced. This process was used to prevent disturbance due to the opening of flasks during fermentation.

2.3.5. Statistical Analysis

The experimental data were analyzed using the Statistical Analysis System (SAS Enterprise Edition v 3.7, SAS Institute Inc., Cary, NC, USA). Data from experiments were compared using Tukey's studentized range (HSD) test at a 5% significance level.

3. Results and Discussion

3.1. Alkaline Treatment for Tannin Removal

A mass balance was performed to investigate the effects of the alkaline tannin removal process on the total weight loss and starch contents of the treated sorghum (Figure 1). The alkaline treatment for tannin removal resulted in an average weight loss of 2.25% on a dry weight basis (dwb), with a concomitant increase in starch content of 1.23% (starch content of 67.5% and 69.9% dwb for untreated and treated sorghum, respectively).

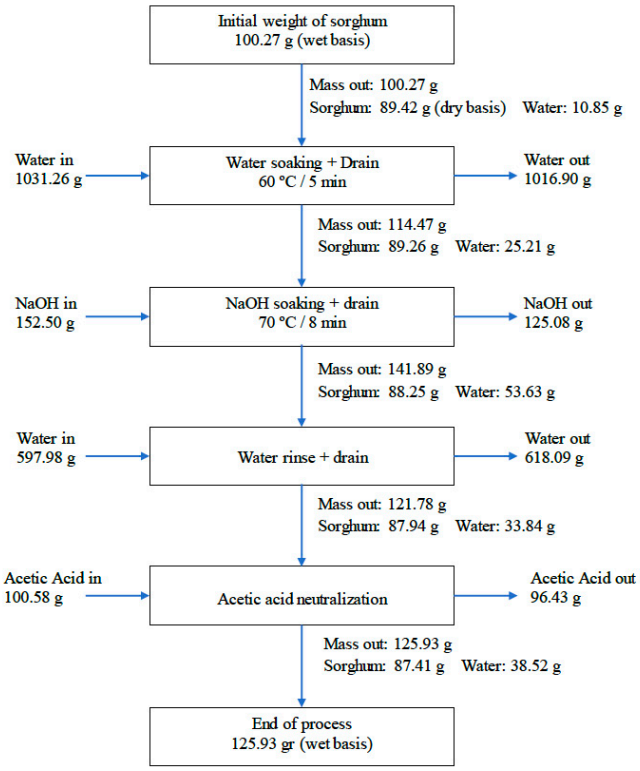

Figure 1. Alkaline tannin removal mass balance.

The tannin content values of the untreated sorghum vs. the final treated sorghum were 266.37 ± 24.49 and 33.77 ± 15.28 mg tannin as cyanidin equivalents/100 g of sorghum (dwb), respectively. These results indicate the advantage of NaOH treatment for tannin removal. This treatment was very efficient and resulted in the removal of more than 80% of the original tannin in the sorghum grains. Water soaking also removed some tannin, but the amount removed was only minimal. In addition, the NaOH treatment did not result in any loss of starch, which is the main substrate for the subsequent ethanol fermentation.

3.2. Fermentations with No Cellulase Added

The fermentation of 100% corn samples was used to determine a baseline for comparisons with treated and untreated sorghum samples (Table 1). The mean ethanol concentration at the end of the 96 h fermentation of corn was 9.39 ± 0.14 % *w/v*.

Table 1. Corn and sorghum fermentations, no cellulase added.

Experiment	Mean Ethanol (% *w/v*) at 96 h Fermentation Time
100% Corn	9.39 ± 0.14
100% Treated sorghum	9.39 ± 0.26
100% Untreated sorghum	8.02 ± 0.15

Since corn, untreated sorghum, and treated sorghum have different starch contents, the theoretical yield values must be used to compare fermentation efficiency. The theoretical yields shown in Table 2 were calculated as shown in Appendix A. The ethanol produced with corn represented an average theoretical yield of 76.79% and was set as a comparison parameter.

Table 2. Average theoretical yield (%) of sorghum fermentations, no cellulase added.

Treatment	Theoretical Ethanol Production (mL)	Ethanol Produced (mL)	Theoretical Yield (%)
100% Corn	12.83	9.85	76.79%
100% Treated sorghum	12.56	9.85	78.48%
100% Untreated sorghum	12.13	8.26	68.15%

When comparing the theoretical yields obtained for this experiment, there was a significant increase ($p < 0.0001$) for 100% treated sorghum, when compared with 100% untreated sorghum. The theoretical yield increased from 68.15 ± 1.46% to 78.48 ± 2.47%. Tannin removal by alkaline pretreatment resulted in an increase in ethanol concentration to a level that was equivalent to the baseline of corn, being 76.79 ± 1.27% theoretical yield. The lower quantity of ethanol produced when using untreated sorghum may be related to the protein-binding capabilities of tannins that triggered reduced enzyme activity. This is consistent with the observations made by Awika et al. [16] and Nkomba et al. [17] regarding tannins interfering with digestive enzyme activity.

Similar results were obtained [18] when ozone-treated sorghum flour was compared with untreated sorghum flour for ethanol fermentation. The ethanol yield from ozone-treated tannin grain sorghum was approximately 90%, which was 8–14% higher than that of untreated samples after 36 h of fermentation. The tannin concentration of ozone-treated sorghum flour decreased significantly from 3.8 to 2.7% after tannin removal treatment [18].

The mean ethanol values obtained from the fermentation of mixtures of corn and treated sorghum are shown in Table 3.

Table 3. Ethanol generated by corn and sorghum mixtures, no cellulase.

Experiment	Mean Ethanol (% *w*/*v*) at 96 h Fermentation Time
75% Corn + 25% Treated sorghum	9.83 ± 0.18
50% Corn + 50% Treated sorghum	9.68 ± 0.17
25% Corn + 75% Treated sorghum	9.71 ± 0.18

As shown in Table 4, the fermentation of the 25, 50, and 75% treated sorghum mixtures improved the theoretical yield when compared with 100% corn or 100% untreated sorghum.

Table 4. Theoretical yield (%) for mixtures of corn and treated sorghum, no cellulase.

Treatment	Theoretical Ethanol Production (mL)	Ethanol Produced (mL)	Theoretical Yield (%)
25% Treated sorghum + 75% Corn	127.60	103.78	81.33%
50% Treated sorghum + 50% Corn	126.93	102.05	80.40%
75% Treated sorghum + 25% Corn	126.26	102.35	81.07%

The results of the percent theoretical yield for all of the non-cellulase fermentations can be seen in Figure 2. When comparing all fermentations, there was at least one mean that was statistically different from the rest ($p < 0.0001$). The mean ethanol values for the 25, 50, 75, and 100% treated sorghum fermentations were not significantly different. These experiments produced the highest theoretical yield of ethanol. This finding may be economically beneficial to an industrial bioethanol facility because it could enable maximum ethanol production over a range of corn/sorghum mixtures. For example, if corn prices were high, the use of alkaline tannin removal could allow the plant to operate with up to 100% treated sorghum in their mashing procedure, thereby making use of a cheaper feedstock. On the other hand, if corn was cheaper than sorghum, the plant could run with 25% treated sorghum and still have better yields of ethanol when compared with the use of 100% corn or 100% untreated sorghum alone.

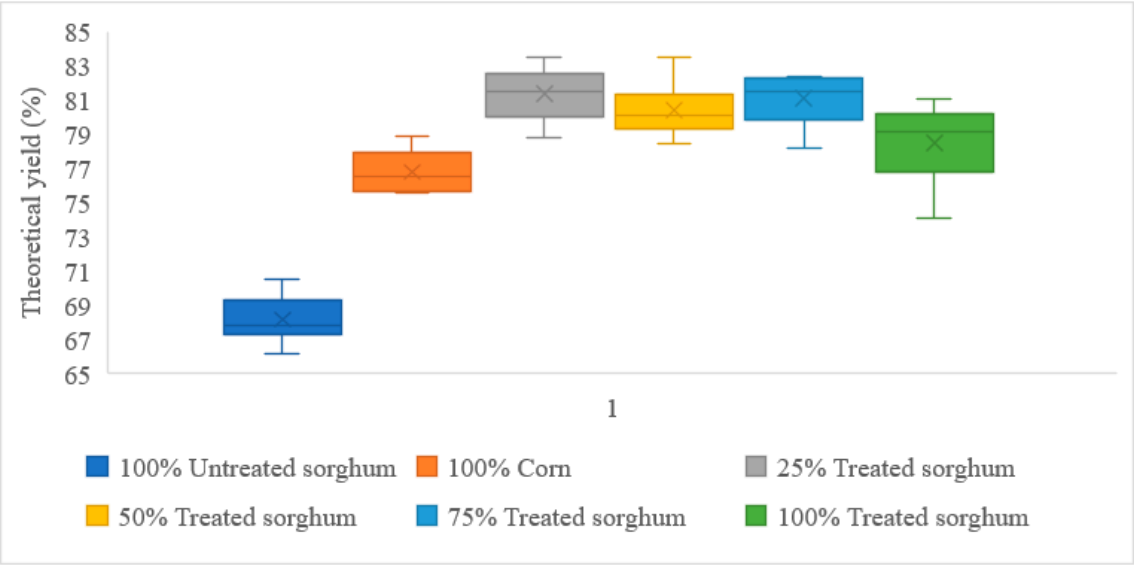

Figure 2. Theoretical yield of non-cellulase fermentations.

3.3. Fermentations with Added Cellulase

The baseline fermentation using corn with added cellulase produced an average of 11.03 ± 0.13 % *w/v* ethanol after 96 h of fermentation (Table 5). The fermentation of treated and untreated sorghum with added cellulase resulted in an increase in ethanol concentration from 8.77 ± 0.18 to 11.29 ± 0.21% *w/v*.

Table 5. Corn and sorghum fermentations with cellulase.

Experiment	Mean Ethanol (% *w/v*) at 96 h Fermentation Time
100% Corn	11.03 ± 0.13
100% Treated sorghum	11.29 ± 0.21
100% Untreated sorghum	8.77 ± 0.18

When comparing average theoretical yields, the corn baseline resulted in 89.81 ± 1.16% of the theoretical yield (Table 6). The 100% untreated sorghum resulted in an ethanol production of 69.77 ± 1.65% of the theoretical yield, whereas for 100% treated sorghum, this value was 94.61 ± 1.93%.

Table 6. Average theoretical yield of sorghum fermentations with cellulase.

Treatment	Theoretical Ethanol Production (mL)	Ethanol Produced (mL)	Theoretical Yield (%)
100% Corn	131.87	118.43	89.81%
100% Treated sorghum	128.64	121.70	94.61%
100% Untreated sorghum	130.61	91.13	69.77%

Similar to treated and untreated sorghum fermentation without cellulase, the alkaline tannin removal treatment significantly improved the percentage of the theoretical yield achieved in fermentation of sorghum ($p < 0.0001$).

The use of cellulase helped to increase the yield of ethanol in all three cases. This may be related to the ability of CTec2® (mixture of cellulase, hemicellulase, and beta-glucosidase) to lower the viscosity of the mixture during liquefaction and fermentation. Decreased viscosity of the mixture resulted in higher enzyme activity, and therefore improved yields on fermentation.

The effect of tannin removal and cellulase addition on ethanol produced by fermentation can be seen in Table 7.

Table 7. Corn and sorghum fermentations with cellulase.

Experiment	Mean Ethanol (% *w/v*) at 96 h Fermentation Time
25% Treated sorghum + 75% Corn	10.93 ± 0.22
50% Treated sorghum + 50% Corn	11.22 ± 0.15
75% Treated sorghum + 25% Corn	11.19 ± 0.19

The theoretical ethanol yield in this experiment increased when compared with non-cellulase treatment (Table 8). According to Tukey's studentized range, the mean theoretical yield values were not statistically different for 50 and 75% treated sorghum, but those values were different from 25% treated sorghum.

Table 8. Average theoretical yield of sorghum fermentations with cellulase added.

Treatment	Theoretical Ethanol Production (mL)	Ethanol Produced (mL)	Theoretical Yield (%)
25% Treated sorghum + 75% Corn	131.06	117.15	89.38%
50% Treated sorghum + 50% Corn	130.25	120.82	92.76%
75% Treated sorghum + 25% Corn	129.44	120.45	93.05%

The mean values obtained for theoretical yield were 89.39 ± 1.85% for 25% treated sorghum, 92.76% ± 1.31% for 50% treated sorghum, and 93.05 ± 1.57% for 75% treated sorghum (Table 8).

The results of the theoretical yield for the fermentations when cellulase was added can be seen in Figure 3. When comparing these fermentations, there was at least one mean that was statistically different from the rest ($p < 0.0001$).

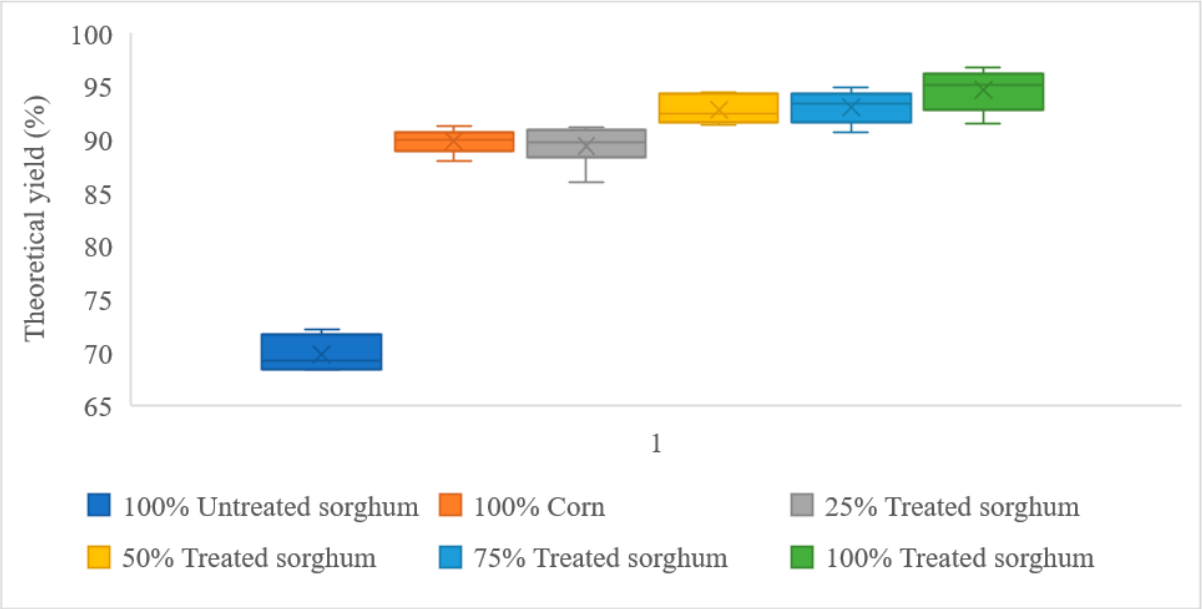

Figure 3. Theoretical yield of fermentations with cellulase.

Comparison of the trials with and without added cellulase indicated that cellulase experiments with treated sorghum resulted in yields that were higher than those without cellulases. This may be related to the effectiveness of CTec2® enzymes in lowering the viscosity of the mixture and improving the efficiency of the other enzymes. In addition, the alkaline tannin removal may act as a pretreatment for lignin removal, exposing even more cellulose to the activity of CTec® enzymes and contributing to a higher ethanol production. The yeast may be fermenting glucose derived from starch hydrolysis and additional glucose derived from cellulose hydrolysis. A comparison of the amount of CO_2 produced during the fermentation of 100% treated sorghum with and without added cellulase addition is shown in Figure 4. As a growth-associated product of ethanol fermentation, CO_2 production is directly related to ethanol production, so was tracked in this study.

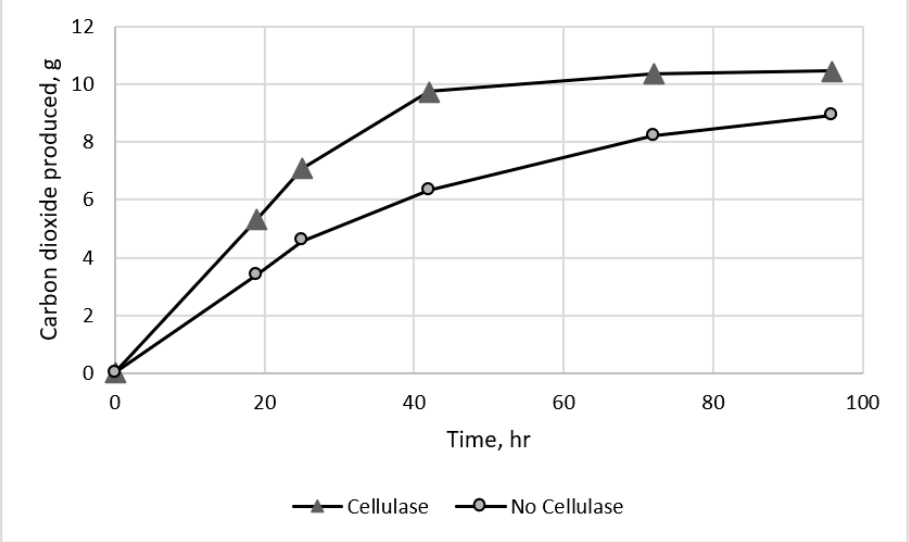

Figure 4. Mean CO_2 produced for treated sorghum with and without cellulase addition (n = mean 6).

4. Conclusions

Alkaline tannin removal pretreatment was effective in removing 87.6% of the tannins of sorghum with only a 2.25% loss (dry basis) and a 1.23% increase in starch availability. The use of alkaline tannin removal resulted in a significant increase in theoretical yield obtained from fermentations using sorghum. The theoretical yield using sorghum when no cellulase was added increased from 68.15 ± 1.46% to 78.48 ± 2.47%, when compared with the corn baseline of 76.79 ± 1.27%. In these non-cellulase experiments, the highest ethanol production was obtained with 25, 50, 75, or 100% treated sorghum mixed with corn.

The combination of cellulase and alkaline tannin removal improved the yield of ethanol in all cases compared to the experiments without cellulase. The highest theoretical yield was obtained when using 50, 75, or 100% treated sorghum mixed with corn when cellulase was added, with an average value of 93.4%.

Overall, tannin removal by alkaline pretreatment was an effective process to remove tannins from sorghum to produce bioethanol at a laboratory scale. Further study is recommended to determine the feasibility and process economics at a larger scale.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Calculation of Theoretical Ethanol Production from Starch

In the experiments, each flask contained 100 g of mash with 25% solids. In each flask, there was 25 g of dry matter and 75 g of water. Considering the starch content of

corn as 71.4% and treated sorghum as 69.9%, both values in dry basis, and assuming a 50% corn + 50% sorghum mixture, the total of fermentable sugars was calculated as:

$$25\ g \times [(50\% \times 71.4\%) + (50\% \times 69.9\%)] = 17.66\ g$$

The amount of glucose produced was calculated from starch hydrolysis stoichiometry as follows:

$$17.66\ g \times 1.111 = 19.62\ g$$

The theoretical ethanol production was calculated from the fermentation stoichiometry as follows:

$$19.62\ g \times 0.511 = 10.03\ g = 12.7\ mL$$

Water consumption during this process was calculated as follows:

$$17.66\ g \times 0.111 = 1.96\ g = 1.96\ mL$$

The final water volume in the flask was:

$$75\ mL - 1.96\ mL = 73.04\ mL$$

Ethanol production will produce a volume increase as follows, with V_F = final volume and V_{EtOH} = ethanol volume, mL:

$$V_F = 73.04 + V_{EtOH}$$

The HPLC ethanol concentration for this example was 9.684% *w/v*. Then:

$$\frac{0.79 \times V_{EtOH}}{V_F} = 0.09684$$
$$V_F = 8.1577 \times V_{EtOH}$$

Substituting,

$$8.1577 \times V_{EtOH} = 73.04 + V_{EtOH}$$
$$V_{EtOH} = 10.20\ mL$$

The mass of ethanol was calculated as follows:

$$10.20\ \ mL\ \times\ 0.79 \frac{g}{mL} = 8.06\ g$$

Finally, the theoretical yield of ethanol for the fermentation was:

$$\frac{8.06\ g}{10.03\ g} \times 100 = 80.36\%$$

For the experiments using cellulase, the value of cellulose present in the feedstock was added to the starch value to calculate the fermentation efficiency.

References

1. Solomon, B.D.; Barnes, J.R.; Halvorsen, K.E. Grain and cellulosic ethanol: History, economics, and energy policy. *Biomass Bioenergy* **2007**, *31*, 416–425. [CrossRef]
2. Balat, M.; Balat, H.; Oz, C. Progress in bioethanol processing. *Prog. Energy Combust. Sci.* **2008**, *34*, 551–573. [CrossRef]
3. Ahmetović, E.; Martín, M.; Grossmann, I.E. Optimization of Energy and Water Consumption in Corn–based Ethanol Plants. *Ind. Eng. Chem. Res.* **2010**, *49*, 7972–7982. [CrossRef]
4. Crago, C.L.; Khanna, M.; Barton, J.; Giuliani, E.; Amaral, W. Competitiveness of Brazilian sugarcane ethanol compared to US corn ethanol. *Energy Policy* **2010**, *38*, 7404–7415. [CrossRef]
5. Drapcho, C.M.; Nghiem, N.P.; Walker, T.H. *Biofuels Engineering Process Technology*; McGraw Hill professional; McGraw-Hill Education: New York, NY, USA, 2007; ISBN 9780071509923.
6. Taylor, J.R.N.; Schober, T.J.; Bean, S.R. Novel food and non-food uses for sorghum and millets. *J. Cereal Sci.* **2006**, *44*, 252–271. [CrossRef]

7. U.S. Grain Council. *2016/2017 Corn Harvest Quality Report and 2016/2017 Sorghum Harvest Quality Report*; U.S. Grain Council: Washington, DC, USA, 2016.
8. Beringer, J.; Bing, J.; Wei, C.; Coram, T.; Curtis, T.; Jim, J.; Elikana Anami, S.; Gabor, G.; Nigel, N.; Jones, H. *Biotechnology of Major Cereals*; CABI: Wallingford, UK, 2016; ISBN 9781780645193.
9. Donke, A.; Nogueira, A.; Matai, P.; Kulay, L. Environmental and Energy Performance of Ethanol Production from the Integration of Sugarcane, Corn, and Grain Sorghum in a Multipurpose Plant. *Resources* **2016**, *6*, 1. [CrossRef]
10. Chuck-Hernandez, C.; Peralta-Contreras, M.; Bando-Carranza, G.; Vera-Garcia, M.; Gaxiola-Cuevas, N.; Tamayo-Limon, R.; Cardenas-Torres, F.; Perez-Carrillo, E.; Serna-Saldivar, S.O. Bioconversion into ethanol of decorticated red sorghum (*Sorghum bicolor* L. Moench) supplemented with its phenolic extract or spent bran. *Biotechnol. Lett.* **2012**, *34*, 97–102. [CrossRef] [PubMed]
11. Barcelos, C.A.; Maeda, R.N.; Betancur, G.J.V.; Pereira, N. Ethanol production from sorghum grains [*Sorghum bicolor* (L.) moench]: Evaluation of the enzymatic hydrolysis and the hydrolysate fermentability. *Braz. J. Chem. Eng.* **2011**, *28*, 597–604. [CrossRef]
12. Wang, D.; Bean, S.; McLaren, J.; Seib, P.; Madl, R.; Tuinstra, M.; Shi, Y.; Lenz, M.; Wu, X.; Zhao, R. Grain sorghum is a viable feedstock for ethanol production. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 313–320. [CrossRef] [PubMed]
13. Johnston, D.J.; Moreau, R.A. A Comparison Between Corn andGrain SorghumFermentation Rates, Distillers Dried Grains with Solubles Composition, and Lipid Profiles. *Bioresour. Technol.* **2017**, *226*, 118–124. [CrossRef] [PubMed]
14. Armstrong, W.D.; Rogler, J.C.; Featherston, W.R. Effect of tannin extraction on the performance of chicks fed bird resistant sorghum grain diets. *Poult. Sci.* **1974**, *53*, 714–720. [CrossRef]
15. Top, S.M.; Preston, C.M.; Dukes, J.S.; Tharayil, N. Climate Influences the Content and Chemical Composition of Foliar Tannins in Green and Senesced Tissues of Quercus rubra. *Front. Plant Sci.* **2017**, *8*, 432. [CrossRef] [PubMed]
16. Awika, J.M.; Rooney, L.W. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* **2004**, *65*, 1199–1221. [CrossRef] [PubMed]
17. Nkomba, E.Y.; van Rensburg, E.; Chimphango, A.F.A.; Görgens, J.F. The influence of sorghum grain decortication on bioethanol production and quality of the distillers' dried grains with solubles using cold and conventional warm starch processing. *Bioresour. Technol.* **2016**, *203*, 181–189. [CrossRef] [PubMed]
18. Yan, S.; Wu, X.; Faubion, J.; Bean, S.; Cai, L.; Shi, Y.-C.; Sun, X.; Wang, D. Ethanol-Production Performance of Ozone-Treated Tannin Grain Sorghum Flour. *Cereal Chem.* **2012**, *89*, 30–37. [CrossRef]