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Franco E. Foglia
Clemson University, francofoglia@gmail.com

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EVALUATION OF EFFECTIVENESS OF TANNIN REMOVAL BY ALKALINE PRETREATMENT ON SORGHUM FOR ETHANOL PRODUCTION BLENDED WITH CORN

A Thesis
Presented to
the Graduate School
of Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biosystems Engineering

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by
Franco E. Foglia
August 2018

Accepted by:
Dr. Caye Drapcho, Committee
Chair Dr. John Nghiem
Dr. Terry Walker

ABSTRACT

Sorghum has been proposed to be a complement to corn for ethanol production. Several advantages have been identified while using sorghum in agricultural and technological aspects. One of the differences between sorghum and corn is the presence of tannins. These compounds are well known for binding proteins and especially affecting enzymatic activity. This is the main disadvantage that sorghum has for ethanol production. High tannin sorghum hybrid XM217 was used to analyze the effect of tannin removal by alkaline pretreatment of sorghum for ethanol production. In this process, 87.6% of the tannins of sorghum were removed. A laboratory-scale dry milling process was used to generate the mashes to be fermented with *Saccharomyces cerevisiae*. Several ratios of corn and treated sorghum were tested, which included 0, 25, 50, 75 and 100% treated sorghum. A fermentation experiment using 100% untreated sorghum also was performed to obtain base-line results. The use of alkaline tannin removal generated a significant increase on the ethanol production compared to the untreated sorghum. The average theoretical yield increased from 68.2 ± 1.5% to 78.5 ± 2.5%, also average ethanol concentrations increased from 8.02 ± 0.15 to 9.39 ± 0.26 % w/v. Mixtures of 25, 50 and 75 and 100% treated sorghum produced the highest ethanol compared to the use of only corn or untreated sorghum. Cellulase was added to a similar set of experiments to determine the feasibility of the tannin removal treatment as a pretreatment method for cellulosic ethanol production. When using alkaline tannin removal with cellulase, a significantly higher ethanol production can be found compared to non-cellulase

experiments. For 100% treated and 100% untreated sorghum trials, the average theoretical yield increased from 69.8 ± 1.7% to 94.6 ± 1.9%, also average ethanol concentrations improved from 8.77 ± 0.18 to 11.29 ± 0.21 % w/v.

DEDICATION

I'd like to dedicate this thesis to Marta, my mom, for her love and support regardless the distance. Also, to Juan Carlos, my dad, whose memories are always present and guiding me. Finally, to my family and friends, because without their support and encouragement this experience would not have been possible.

ACKNOWLEDGMENTS

I would like to first and foremost thank my advisor Dr. John Nghiem for all his patience, support and guidance while working on this thesis. I also want to thank Dr. Caye Drapcho and Dr. Terry Walker, for their support and inspiration.

Thanks to the Argentinian Government through the Bec.AR program and the Argentine Fulbright Commission for supporting me in this process.

Lastly, a special thank you to the people I've had the opportunity to get to know during my experience here at Clemson: this process has been memorable with you all.

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LITERATURE REVIEW

1.1.Fuel consumption

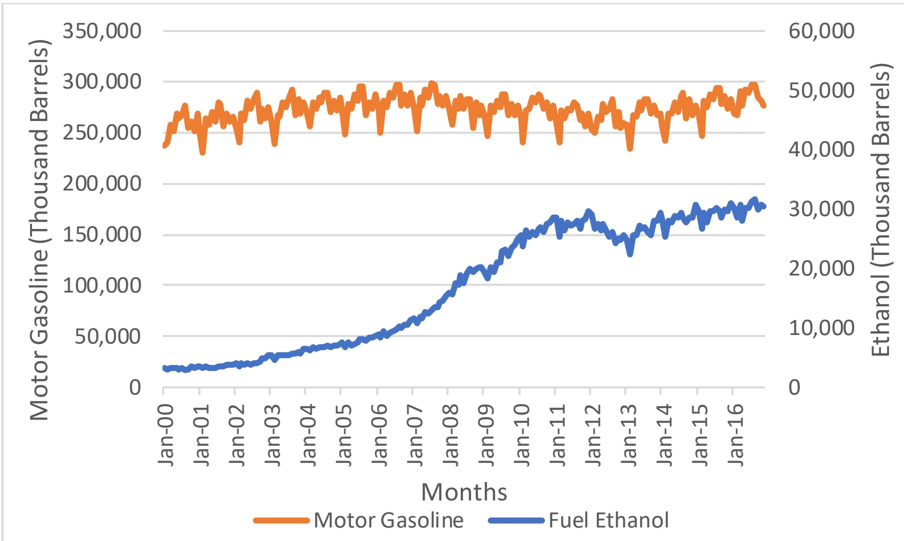


Figure 1. Motor gasoline and Fuel ethanol production[1]

Gas consumption in the United States is growing. In 2016, motor fuel consumption was 3,124,615 thousand barrels (496,774 m^3) according to the U.S. Energy Information Administration (2017b) – EIA. Considering the gas as a non-renewable product, the need to replace it arises. In that situation, bioethanol has been used to reduce the dependence on fossil fuels. For the same period the bioethanol production was 333,396 thousand barrels (53,005 m^3) (U.S. Energy Information Administration, 2017a). The trend of both gas consumption and bioethanol is upgoing, as it can be seen in Figure 1. Ethanol produced

[1] Author's own elaboration based on data of EIA.

accounted for more than 10% of gasoline in volume between 2014 and 2016 according to the information provided by EIA.

1.2.Bioethanol production

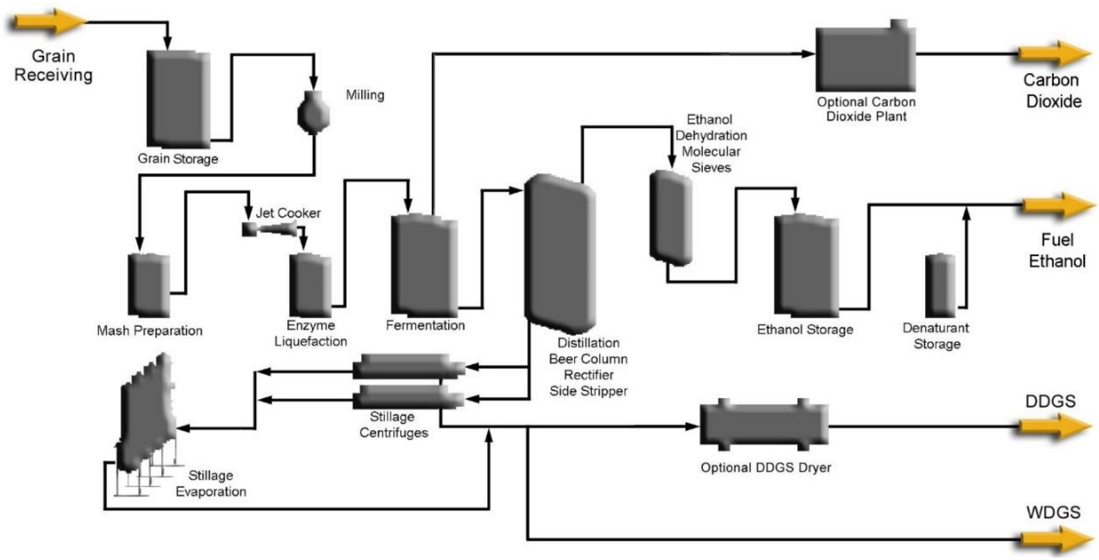


Figure 2. Ethanol production process[2]

An overview of the ethanol production process can be seen in Figure 2. This process, also called as dry milling, starts with a grain storage system. From that point incoming grain is passed thru screens and sifters to eliminate foreign material and potential hazards to equipment. Then, it is sent to a milling process in which the grain will obtain a coarse flour consistency. Hammer or roller mills can be used for this purpose.

[2] Kohl (2005)

After that, corn flour is mixed with hot water and alpha amylase in the process called “mash preparation”. The objective of this step is to start to liquefy starch into dextrins to facilitate pumping and downstream processing. The following phase is an enzymatic liquefaction, that holds this slurry for 60 to 180 minutes to give alpha amylase retention time to keep breaking starch into smaller molecules.

After liquefaction, the mash is cooled at the fermentation temperature and yeast is added and pumped into a fermenter (Bothast & Schlicher, 2005). Also, glucoamylase is added to keep breaking starch, dextrins and maltose to generate readily fermentable glucose. Urea is incorporated as a nitrogen source. Once this mash is in the fermenter, yeast will grow and convert glucose to ethanol, carbon dioxide and heat. Temperature must be controlled to assure proper fermentation conditions to yeast. The fermentation lasts up to 75 hours depending on the facility, being a normal value between 40 and 60 hours (Kohl, 2005).

Once fermentation is over, the obtained product is called “beer” formed by grain solids (fermentable and non-fermentable), water, ethanol and minor components (acetic acid, glycerol, lactic acid, etc.). The beer is pumped into distillation columns which will allow to separate 190 proof ethanol (95% v/v) from water and solids.

190 proof ethanol is pumped into molecular sieves to remove the 5% remaining water, obtaining 200 proof ethanol or 99.5% v/v (Walker, 2012). Solids and water after distillation will be further processed by using centrifuges and evaporation to produce WDG and syrup that when mixed produce WDGS (Wet Distiller’s Grains with Solubles). An

alternative process can be applied to dry WDGS into DDGS (Dried Distiller's Grains with Solubles) that improve the shelf life of the product.

The process described in Figure 2 is water demanding and energy consuming. Water consumption varies from 3 to 15 gallons of water per gallon of ethanol produced (Ahmetović, Martín, & Grossmann, 2010). On the other hand, energy demand can be seen in WDGS drying (DDGS production) where it requires approximately one-third of the total energy consumed for the plant (Bothast & Schlicher, 2005; Mosier & Ileleji, 2015). The same authors also claim that a high-quality DDGS contributes to the profitability of the plant compared to WDGS.

Sustainability is a key challenge to bioethanol production. By knowing this, alternatives that can reduce the energy consumed or increase ethanol production must be considered. One of the alternatives is the use of sorghum instead of corn. This option will allow to locally grow sorghum and reduce CO2 emissions related to corn transportation. Also, sorghum has a similar grain composition than corn and it can be used interchangeably with corn in a dry-grind corn ethanol plant with few or minimum engineering modifications.

A second alternative found in literature is an integrated process (Vander Griend, 2009). This patent describes how to integrate several processes to minimize water consumption, steam and energy. Some of the actions described in the patent are: recycle part of thin stillage to cook (reduce water consumption), use steam from evaporation of thin stillage for distillation processes, use of two or more effects on thin stillage concentration (allowing to reduce the total amount of boiler steam), use of condensate from

the effects in the cook process, use evaporators to condensate 200 proof ethanol, among others. Ahmetovic et al. (2010), have discussed several optimization methods for ethanol plants that involve recycle and reuse of process and cooling water and steam.

A third option to make this process more sustainable is the use of corn in the maximum amount possible. The patent analyzed, claims the opportunity to integrate a cellulosic fuel process to a traditional bioethanol plant (Javers et al., 2017). This process allows better ethanol yields with the same amount of corn. Reducing the amount of corn that needs to be planted also transportation, and therefore CO2 emissions.

1.3.Feedstocks for ethanol production

Currently in the US, 30% of the corn harvested is being used to produce bioethanol (Crago, Khanna, Barton, Giuliani, & Amaral, 2010). This represents 95% of the raw material used in this process, the rest is made from wheat, barley, cheese whey and beverage residues (Drapcho, Nghiem, & Walker, 2007; Solomon, Barnes, & Halvorsen, 2007).

The use of corn as a raw material for the production of bioethanol has been criticized because of soil erosion, loss of biodiversity, high use of nitrogen fertilizer, significant use of land and water and having a negative CO_2 balance (Ahmetović et al., 2010; Balat, Balat, & Oz, 2008; Solomon et al., 2007). This last critic has been challenged by Hammerschlag (2006) that claims that the production of bioethanol result in a net reduction of CO_2 emissions and it can replace fossil fuel use. Besides these critics, the use of corn for bioethanol production derived in a controversy. This is called "food vs fuel

controversy". Several authors have claimed that the use of corn to produce biofuels has raised the cost of corn-based or corn-related products. This affects the population, especially low-income and malnourished families. One author states "ethanol production could entail diverting valuable cropland from producing corn needed to feed people to producing corn for ethanol factories" (Pimentel & Patzek, 2005).

There are several alternatives to face this problem. The first one is to use a production process called "second generation ethanol" or "cellulosic ethanol" that uses residual non-food parts of current crops or crops not used for food purposes. Second generation ethanol can be made of wood chips, wood residues, paper, sewage sludge, municipal residues and cereal straws among others (Solomon et al., 2007). The problem that arises with the use of second generation ethanol is to extract the sugars from the complex and diverse chemical structures present in the several types of feedstocks. A good example of this is the use of woody or fibrous materials which contain cellulose, hemicellulose and lignin. Several options have been developed to hydrolyze these molecules into its monomers. Such processes include, but are not limited to, enzymatic hydrolysis, steam heating, pyrolysis and chemical pre-treatments. Unfortunately, second generation ethanol is not commercially spread, mainly due to excessive costs.

A second alternative to meet this problem is the use of non-food crops that are rich in starch. Sorghum has been proposed to be a complement or replacement for corn (Taylor, Schober, & Bean, 2006).

1.4.Sorghum

Sorghum bicolor commonly called sorghum or also known as milo, is an important cereal with a production of 639,30,558 ton in 44,771,056 hectares in 2016 (Food And Agriculture Organization (FAO), 2016). Grain sorghum (Sorghum bicolor ssp. Bicolor) is cultivated to produce grains mainly for livestock feed and for ethanol production in a minor way. Also, some varieties can produce green chop, hay, silage, and pasture if stems and foliage are used.

Sorghum does not contain gluten or its constituents: gliadins and glutenins. This makes it a suitable candidate to replace wheat, oats, barley and rye from the diets of people with celiac disease. Nowadays it can be found in supermarkets sorghum flour and sorghum syrup (sweet sorghum). Both product can be easily consumed by celiac population. Gluten-free bread from sorghum requires the use of a different technology than wheat bread. This is why it is important to select the right hybrid to produce the flour (Schober, Messerschmidt, Bean, Park, & Arendt, 2005) and also the right technology (Schober, Bean, & Boyle, 2007).

Some varieties of sorghum are also used for broom production. Sorghum bicolor var. Technicum can be used as an example of the varieties suitable for this use (Estrada et al., 2012).

The production of grain sorghum in the United States is mainly done in the states of Kansas, Texas, Colorado, Oklahoma, South Dakota and Louisiana. According to information of the United States Department of Agriculture (2017), during 2017 2,276,762

hectares were planted with grain sorghum producing 10,187,296 tons with a yield of 4.5 ton/ha.

The use of sorghum has several advantages for the ethanol production process. The first one is that sorghum has a similar composition as corn, especially in starch. Table 1 shows the composition of corn and sorghum. A second feature of sorghum is its agricultural conditions: it is well-adapted to environments with high temperature and water limitations and also tolerant to drought stress (Beringer et al., 2016; Donke, Nogueira, Matai, & Kulay, 2016). This is an advantage because sorghum can be an alternative to be grown in environments where corn isn't, especially in dry areas (Barcelos, Maeda, Betancur, & Pereira, 2011; Chuck-Hernandez et al., 2012; Taylor et al., 2006).

Table 1. Composition comparison between corn and sorghum

PARAMETERS	**2016 Harvest CORN**			**2016 Harvest Sorghum**		
	No. of Samples	Avg.	Std. Dev.	No. of Samples	Avg.	Std. Dev.
Protein (dry basis, %)	624	8.6	0.50	246	8.5	1.10
Starch (dry basis, %)	624	72.5	0.59	246	72.6	0.91
Oil (dry basis, %)	624	4.0	0.23	246	4.4	0.25

(U. S. Grain Council, 2016a, 2016b)

1.5. Tannins

Tannins are chemical compounds derived from tannic acid also known as phenolic acid. Tannins are widely spread through plants, especially among trees. As an approximation, tannins can be found in 80% of the woody perennial dicotyledons. This

number decreases to 15% of annual and herbaceous perennial dicotyledon species (Combs, 2016). They can be classified in two groups according to their structures: proanthocyanidins (condensed tannins) and hydrolyzable tannins. Figure 3 shows the chemical structure of sorghum proanthocyanidin.

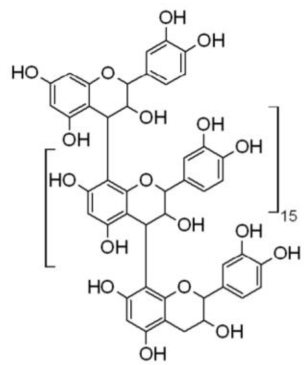


Figure 3. Sorghum proanthocyanidin [3]

Tannins are known for binding to proteins. This lies in the phenolic groups particularly in their structure which facilitates the bonding to carbonyl groups of peptides (Petridis, 2011). In a way, tannins act as a defense mechanism for plants. It is part of a quantitative defense that slows the growth rate of herbivores (Stamp, 2003). Other mechanism related to this one is the astringent sensation when eaten. This is due to the binding of salivary proteins and tannins when eaten (MacAdam, Brummer, Islam, & Shewmaker, 2013). Scalbert (1991) has discussed several mechanisms of tannin toxicity such as enzyme inhibition and substrate deprivation (astringency), action on membranes and complexation of metal ions. Other authors have described that higher levels of tannins

[3] (Halvorson, Gonzalez, Hagerman, & Smith, 2009)

in forages are related to decreased protein utilization, especially because of the presence of tannin-bound proteins in feces (Barbehenn & Peter Constabel, 2011).

The idea of using of sorghum for bioethanol production brings a new problem to face: tannins. Red and white sorghum contain different quantities of condensed tannins. They are located in the testa layer of grain and help to increase the resistance to some insects and weather conditions (Taylor et al., 2006). However, it has been demonstrated that tannins increase the viscosity affecting the activity of amylases (Johnston & Moreau, 2016; Wang et al., 2008)

Several processes have been proposed to remove tannins from sorghum in bioethanol production: decortication (Corredor, Bean, Schober, & Wang, 2006; Johnston & Moreau, 2016; Wang et al., 2008), protease pretreatment (Chuck-Hernandez et al., 2012; Johnston & Moreau, 2016) and steam-flaking (Chuck-Hernandez et al., 2012). Several authors have evaluated alkaline treatments to remove tannins from sorghum for different purposes such as animal feed, milling and protein composition (Ali, El Tinay, Elkhalifa, Salih, & Yousif, 2009; Armstrong, Rogler, & Featherston, 1974; Beta, Rooney, & Taylor, 2000; Blackwell, Herald, Bean, & Gadgil, 2012), but none of those have been tried in dry grind ethanol production.

1.6. Conclusion and goal of research

Energy sources are required for the development of any country. Fossil fuel dependence is a worldwide reality and several efforts are made to migrate to more

sustainable energy sources. In this way, the use of biofuels is a way to mitigate fossil fuel use while protecting the environment.

Bioethanol is an energy source that has been challenged because of land use and being water and energy intensive. In this way, sorghum can contribute to the sustainability of the bioethanol production process. It has been discussed that it has better agricultural conditions than corn, especially a lower water requirement. Some of the challenges that arises with sorghum are tannins and the possibility to bind to proteins. This may be a challenge because the use of enzymes to hydrolyze the starch containing in sorghum to generate fermentable sugars. On the other hand, engineering optimizations are needed to decrease the use of water and steam in a biorefinery that produces ethanol.

The goal of this research is to determine the impact of tannin removal by alkaline pretreatment of sorghum on ethanol production by itself and mixed with corn.

CHAPTER TWO:

MANUSCRIPT

1. Abstract

Sorghum has been proposed to be a complement to corn for ethanol production. Several advantages have been identified while using sorghum in agricultural and technological aspects. One of the differences between sorghum and corn is the presence of tannins. These compounds are well known for binding proteins and especially affecting enzymatic activity. This is the main disadvantage that sorghum has for ethanol production. High tannin sorghum hybrid XM217 was used to analyze the effect of tannin removal by alkaline pretreatment of sorghum for ethanol production. In this process, 87.6% of the tannins of sorghum were removed. A laboratory-scale dry milling process was used to generate the mashes to be fermented with *Saccharomyces cerevisiae*. Several ratios of corn and treated sorghum were tested, which included 0, 25, 50, 75 and 100% treated sorghum. A fermentation experiment using 100% untreated sorghum also was performed to obtain base-line results. The use of alkaline tannin removal generated a significant increase on the ethanol production compared to the untreated sorghum. The average theoretical yield increased from 68.2 ± 1.5% to 78.5 ± 2.5%, also average ethanol concentrations increased from 8.02 ± 0.15 to 9.39 ± 0.26 % w/v. Mixtures of 25, 50 and 75 and 100% treated sorghum produced the highest ethanol compared to the use of only corn or untreated sorghum. Cellulase was added to a similar set of experiments to determine the feasibility of the tannin removal treatment as a pretreatment method for

cellulosic ethanol production. When using alkaline tannin removal with cellulase, a significantly higher ethanol production can be found compared to non-cellulase experiments. For 100% treated and 100% untreated sorghum trials, the average theoretical yield increased from 69.8 ± 1.7% to 94.6 ± 1.9%, also average ethanol concentrations improved from 8.77 ± 0.18 to 11.29 ± 0.21 % w/v.

2. Introduction

Energy sources are required for the development of any country. Fossil fuel dependence is a worldwide reality. Following that global trend, gasoline consumption in the United States is growing. In 2016, motor fuel consumption was more than 3.1 billion barrels (almost 500,000 m^3) according to the U.S. Energy Information Administration (2017b). Several efforts are made to migrate to more sustainable energy sources. One alternative is the use of biofuels such as bioethanol or biodiesel. Both are good options to help mitigate fossil fuel use while protecting the environment and allowing a country to have the energy needed for development. For the same period the bioethanol production was 333,396 thousand barrels (53,005 m3) (U.S. Energy Information Administration, 2017a). In the United States, the most common blend of ethanol and gasoline is E10 (10% ethanol, 90% gasoline). Other options are available such as E85, which is a blend containing 51% to 83% ethanol by volume or E15 (10.5% to 15% ethanol with gasoline) (U.S. Department of Energy, 2016).

Sustainability is a key challenge to bioethanol production. It has been demonstrated that it is a water demanding and energy consuming process. Also, the use

of corn as a raw material to produce bioethanol has been criticized. Soil erosion, loss of biodiversity, high use of nitrogen fertilizer, significant use of land and water and negative CO_2 balance (Ahmetović et al., 2010; Balat et al., 2008; Solomon et al., 2007) are some of the reasons.

Currently in the US, 30% of the corn harvested is used to produce bioethanol (Crago et al., 2010). This represents 95% of the raw material used in this process, the rest is made from wheat, barley, cheese whey and beverage residues (Drapcho et al., 2007; Solomon et al., 2007).

Several options have been analyzed to address these problems. Engineering approaches such as those proposed by Vander Griend (2009) or Ahmetovic et al. (2010) attempt to optimize the resources utilized in a bioethanol plant. Recycle and reuse of process and cooling water, steam and energy are key points proposed by those authors. Another engineering approach developed by Javers et al. (2017) considers the opportunity to integrate a cellulosic fuel process into a traditional bioethanol plant. This process may allow better ethanol yields with the same amount of corn. Reducing the amount of corn that needs to be planted also reduces transportation needs, and therefore CO_2 emissions. An economic analysis is needed to evaluate the feasibility of this process at an industrial scale. The main idea is interesting and could be an alternative for the currently non-economically feasible cellulosic fuels.

Another alternative to meet this problem is the use of non-food crops that are rich in starch. Sorghum has been proposed to be a complement or replacement for corn (Taylor et al., 2006). The use of sorghum has several advantages for the ethanol

production process. The first one is that sorghum has a similar composition as corn, especially in starch. A second feature of sorghum is its agricultural conditions: it is well-adapted to environments with high temperature and water limitations and also tolerant to drought stress (Beringer et al., 2016; Donke et al., 2016). This is an advantage because sorghum can be an alternative to be grown in environments where corn is not, especially in dry areas (Barcelos et al., 2011; Chuck-Hernandez et al., 2012; Taylor et al., 2006). These characteristics allow to locally grow sorghum and reduce CO_2 emissions related to corn transportation. The similar grain composition of corn and sorghum allows it to be used interchangeably with corn in a dry-grind corn ethanol plant with minimum engineering modifications.

One of the differences between corn and sorghum is the presence of tannins in sorghum. Tannins are compounds derived from tannic acid, which is also known as phenolic acid. Tannins are widely spread through plants. Red and white sorghum contain different quantities of condensed tannins. They are located in the testa layer of grain and help to increase the resistance to some insects and weather conditions (Taylor et al., 2006). In a way, tannins act as a defense mechanism for the plants. It is part of a quantitative defense that slows the growth rate of herbivores (Stamp, 2003). Tannins also are known for binding to proteins. This is a challenge in bioethanol production because of the use of enzymes to hydrolyze starch and dextrins to simple sugars. It has been demonstrated that tannins increase the viscosity, affecting the activity of amylases (Johnston & Moreau, 2016; Wang et al., 2008). This represents a major problem in a biorefinery because of loss of ethanol production and agitation and pumping issues.

The objective of this research is to investigate the impact of tannin removal by alkaline pretreatment of sorghum for ethanol production. The goal is to find which combination of corn and treated sorghum produces the highest ethanol yield. Also, it is a good opportunity to evaluate if the tannin removal process is suitable as a pretreatment method for cellulosic ethanol production using a commercial cellulase.

3. Materials and methods

3.1. Materials

No. 2 Yellow Corn, which was grown in Anderson County, SC, was obtained from a local provider (Griff's Farm & Home Center, Pendleton, South Carolina). High-tannin sorghum hybrid XM217, which was 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 subsequently stored in plastic bags in a room with low ambient humidity and at room temperature.

The enzymes used in this research were stored in a refrigerator. Liquozyme SC DS® (α-amylase), Spirizyme Ultra® (glucoamylase) and CTec2® (cellulase) were provided by Novozymes (Franklinton, North Carolina).

Active dry yeast C6 FUEL™ (*Saccharomyces cerevisiae*), was provided by Lallemand Biofuels & Distilled Spirits (Duluth, GA) and stored in the refrigerator.

All chemicals were purchased from VWR (Georgia, USA) and were of analytical grade.

3.2. Methods

3.2.1. Solutions preparation

Liquozyme SC DS® (α-amylase) and Spirizyme Ultra® (glucoamylase) were diluted to a 1:50 (v/v) with de-ionized water and stored in a refrigerator. Also, a 10% w/v solution of urea in de-ionized water was made and kept refrigerated.

3.2.2. Yeast hydration

Active dry yeast was rehydrated before use. A yeast slurry was prepared by mixing 2.5 g of active dry yeast with 50 ml of de-ionized water and stirred for 30 minutes. After this process yeast is ready to be dispensed in fermentation flasks.

3.2.3. Flasks and rubber stoppers sterilization

250-ml flasks were sterilized in a gravity displacement steam sterilizer. Sterilization temperature of 121 ºC was held for 20 minutes and then cooled to room temperature.

3.2.4. Corn and sorghum milling

Corn, 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 and kept in a plastic bag in a freezer. Corn and sorghum for all the experiments were milled at the same time. Moisture content of each bag was determined by drying 2 gr of sample at 103 ºC +/- 2 ºC for 8 hours until constant weight.

3.2.5. Mash liquefaction

Mash was prepared in a 3-liter stainless steel beaker containing 1600 g of mash in total weight. The amount of grains were added in different proportions (100% untreated sorghum, 100% corn, 25% treated sorghum + 75% corn, 50% treated sorghum + 50% corn, 75% treated sorghum + 25% corn and 100% treated sorghum), reaching in all cases 400 g of dry solids. Water was calculated as the amount needed to reach 1600 g of total weight. The solids percentage in mash was 25%.

Slurry was agitated with a mechanical agitator at 500 rpm during the experiment. pH was adjusted to 5.5 by using a 10 N sulfuric acid solution. Liquozyme SC DS® (α-amylase) was added in a dosage of 0.058 g enzyme per 100 g of dry solids (9.22 ml of diluted solution per batch). The mash was kept at 85 ºC by using a water bath on a hot plate. Starch liquefaction lasted 2 hours and it included the addition of small amounts of water to compensate for evaporation during this process. At the end of the liquefaction, the beaker was cooled to 50 ºC in an ice bath. After that, the beaker was weighed, and water was added to obtain 1600 g of total weight. The liquefied slurry was then split into two smaller beakers, containing 800 g of mash in each one.

3.2.6. Non-cellulase fermentation experiments

After producing a liquefied mash, as it can be seen in section 3.2.5, a beaker containing 800 g was cooled further in an ice bath. When the temperature was below 40 ºC, mash was stirred again at 300 rpm, and pH was adjusted to 3.90 – 4.10 by using a 10 N sulfuric acid solution. Spirizyme Ultra® (glucoamylase) was added in a dosage of

0.116 g enzyme per 100 g of dry solids (10.22 ml of diluted solution per 800 g batch) together with 3.20 ml of diluted urea solution. The agitation of the mash continued for 20 minutes to facilitate the dissolution of urea and even distribution of glucoamylase.

Mash was dispensed into 250 ml sterilized flasks at 100 g per flask. Each flask was added of 0.50 ml of yeast slurry prepared as described in 3.2.2 immediately after 30 minutes of hydration was completed. Flasks were capped with #6 rubber stoppers. An 18-gauge hypodermic needle was inserted into the rubber stopper to allow pressure relief from carbon dioxide production in fermentation.

Finally, the flasks were incubated in an orbital shaker at 180 rpm and 32 ºC. Simultaneous saccharification and fermentation was carried out for 96 hours. Flasks were weighed periodically to determine weight loss due to carbon dioxide release, which also was used to follow the progress of ethanol production. Fermentation was considered completed at 96 hours and reaching less than 5% of weight loss in eight hours. Each experiment had six replicates.

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

3.2.7. Cellulase fermentation experiments

After producing a liquefied mash, (see 3.2.5) the 800 g beaker was kept in a water bath at 50 ºC with agitation of 300 rpm. 6 ml of CTec2® were added to the beaker and agitation was continued for 1 hour.

Subsequently, the process was the same as a non-cellullase experiments which can be seen in 3.2.6.

3.2.8. Tannin removal procedure

The tannin removal procedure was modified from Armstrong et al. (1974). Samples were presoaked in deionized water at 60 ºC for five minutes with constant stirring. After draining, sorghum was soaked in 20% sodium hydroxide at 70 ºC for eight minutes. A ratio of 0.80 kg of grain per liter of alkaline solution was used. Then, the grain was let to drain in a metallic screen and rinsed with hot tap water (50 ºC) until pH 8. Finally, samples were dried in a forced air oven at 70 ºC for 6 hours and then stored. The original procedure described in literature included a final neutralization step with acetic acid which was not used in this study. The main reason is related to the harmful effects of small remaining amounts of acetic acid at the start of fermentation.

3.2.9. Tannin mass balance

Three small treatments were used to create the tannin removal procedure mass balance. The amount of grain used in each one was approximately 100 grams wet basis. The tannin removal procedure was used as described on 3.2.8. Samples were collected by triplicate of each step of the process. Finally, tannin content was determined in each one by using the method described in 3.3.1.

3.3. Analytical methods

3.3.1. Condensed Tannins Analysis

The tannin content of sorghum was determined by using the acid-butanol assay modified from Porter et al. (1985) as used by Top et al. (2017). Approximately 50 mg of sorghum sample was weighed into glass tubes and then added 6 ml of the butanol:HCl (95:5 v/v) reagent. Samples were incubated in a water bath at 90–95ºC for an hour, vortexing before and halfway through and then cooled on ice.

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

3.3.2. HPLC analysis

Ethanol concentrations were determined by using a Shimadzu HPLC. The equipment had a mobile phase of 0.005 M sulfuric acid and was operated at 60 ºC with a flow of 0.60 ml/min. The HPLC had an Aminex® HPX-87H 300x7.8 mm ion exclusion column (Bio-Rad Laboratories, CA, USA) and a RID-10A refractive index detector (Shimadzu). The data was collected and analyzed by using LCsolution version 1.25 (Shimadzu Corporation, Japan).

Samples obtained from 3.2.6 and 3.2.7 were analyzed by using this method using two injections per sample and then taking the average of values. A calibration curve was previously developed for all analyzed fermentation products.

3.3.3. Statistical analysis

The experimental data was analyzed using 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.

4. Results and discussion

4.1. Alkaline tannin removal mass balance

A mass balance was performed to investigate how the alkaline tannin removal procedure affected the tannin content of the feedstock, as well as possible weight loss. For the mass balance, the whole procedure was performed as it can be found in literature. For fermentation experiments, the neutralization step was removed. This is due to the negative effects that acetic acid would have at the start of fermentation.

The results of the overall mass balance can be seen in Figure 4. Results are expressed in dry basis unless otherwise specified. Analyzing this figure, it can be said that the process had an average weight loss of sorghum of 2.01 g which represents 2.25% of dry feedstock. Also, when analyzing starch content from untreated and treated sorghum the values found were 67.5% and 69.9% dry basis respectively. This represents a 1.23% of starch increase. This may be related to the removal of non-starch compounds during the tannin removal process and less interference of tannins in the method of starch determination (enzymatic method). The low product loss and starch availability increase suggests that this process may be feasible for industrial use. Further scaled-up

experiments may be useful to determine that feasibility. Also, a process optimization may be required while moving to a pilot or industrial scale.

The retention times used in this process will allow to have small tanks reducing the investment and operating costs (heating, insulation, amount of chemical in each tank) required when adopting this method at a large scale. An important item that helps the sustainability of the process is water use. If the neutralization step with acetic acid is not performed, a higher amount of water would be needed to remove the sodium hydroxide or maybe consider the possibility of using another neutralizing agent. Sulfuric acid is a chemical usually found in an ethanol biorefinery and a diluted solution may help neutralize the grains without the problems associated to acetic acid.

Table 2. Tannin content of the tannin removal mass balance samples

Step	Average tannin content (mg tannin/100 g sorghum)[4]
Original Sample	266.37 ± 24.49
After water soaking	238.23 ± 17.91
After NaOH soaking	48.18 ± 5.70
After final rinse	33.77 ± 15.28

A second finding from this process is the final moisture content of the grains is 30.58%. With this moisture the grains are prone to fungal contamination and

[4] Tannin content expressed as average mg tannin as cyanidin equivalents per 100 g sorghum dry basis

deterioration (Owens, 2001). Also, it may become a problem when milling the grains in a hammer or roller mill. So, it is recommended to dry the grains for further storage and processing.

Table 2 depicts the tannin content in different steps of the process. An interesting outcome of this data is that the water soaking step removed in average 28.57 mg tannin/100 g dry sorghum about 10.7%. Secondly, the most significant tannin drop is after the alkaline step (NaOH soaking), as expected, with an 82.2% tannin reduction compared to the original sample. The overall tannin reduction is 87.6%.

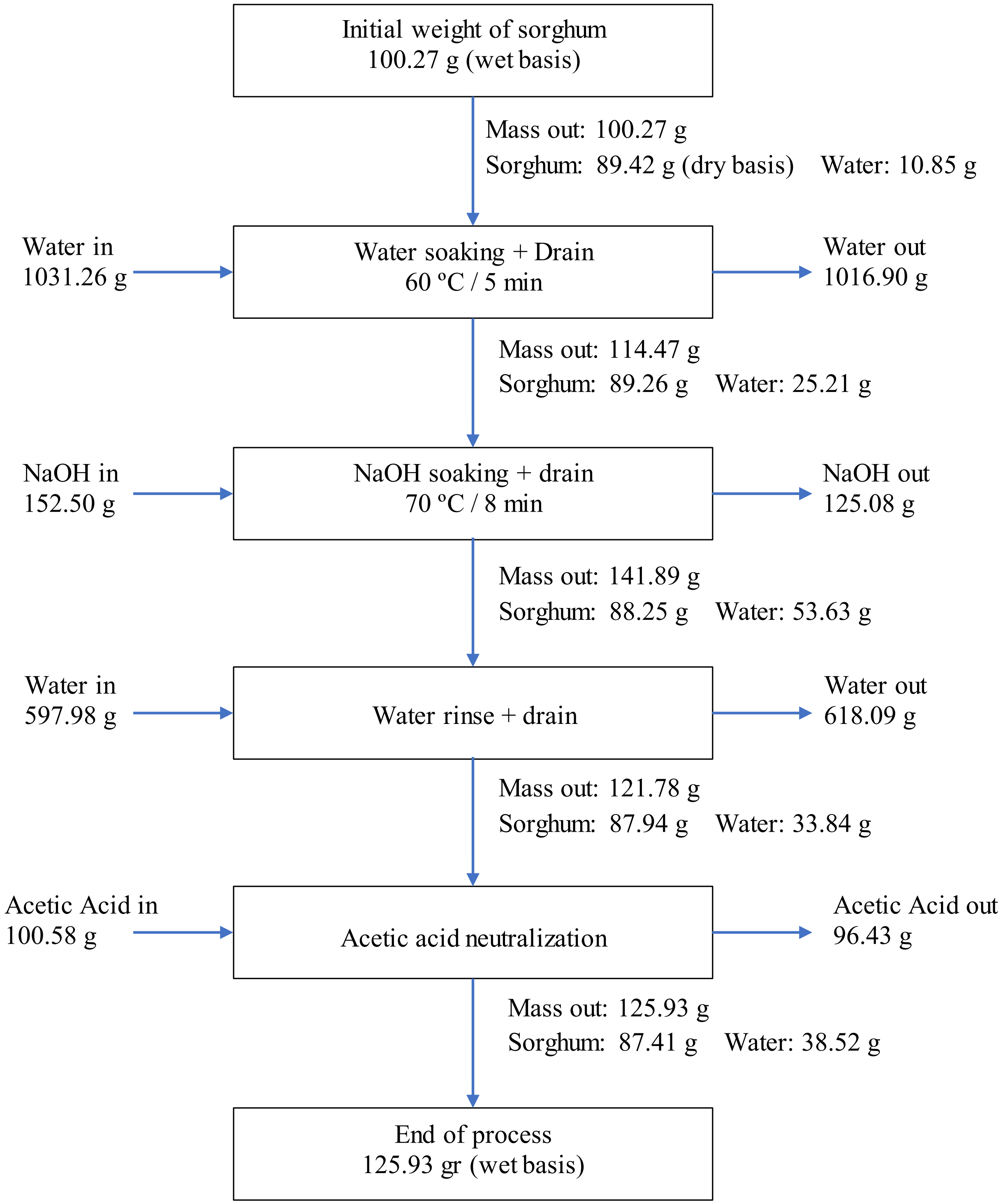


Figure 4. Alkaline tannin removal mass balance

4.2. Non-Cellulase fermentations

4.2.1. Effect of alkaline tannin removal on 100% sorghum fermentation

An experiment of 100% corn was used to determine a baseline for comparisons. In this case, the ethanol produced by corn is 9.39 ± 0.14 % w/v. This value represents an average theoretical yield of 76.79% and will be set as a comparison parameter. The theoretical yield can be calculated as it can be seen in Appendix A. Results of the theoretical ethanol productions and the theoretical yield (%) for this experiment are shown in Table 4. Table 3 shows the ethanol concentration in w/v at the end of a 96 hours fermentation. Since corn, untreated sorghum and treated sorghum have different starch contents theoretical yield must be used to compare fermentation efficiency.

Table 3. Corn and sorghum fermentations, no cellulase

Experiment	Ethanol (% w/v) at 96 h of fermentation time [6]
100% Corn	9.39 ± 0.14
100% Treated sorghum	9.39 ± 0.26
100% Untreated sorghum	8.02 ± 0.15

When comparing theoretical yield obtained for this experiment, there is a significant increase ($P < 0.0001$) when using 100% treated sorghum instead of 100% untreated sorghum. The theoretical yield increased from 68.15 ± 1.46% to 78.48 ± 2.47%. This information can also be seen graphically in Figure 4. It is important to point

[6] Average of six replicates.

out that the tannin removal by alkaline pretreatment, contributed to the increase on ethanol concentration to a level in which it is equivalent to the baseline of corn: 76.79 ± 1.27% theoretical yield.

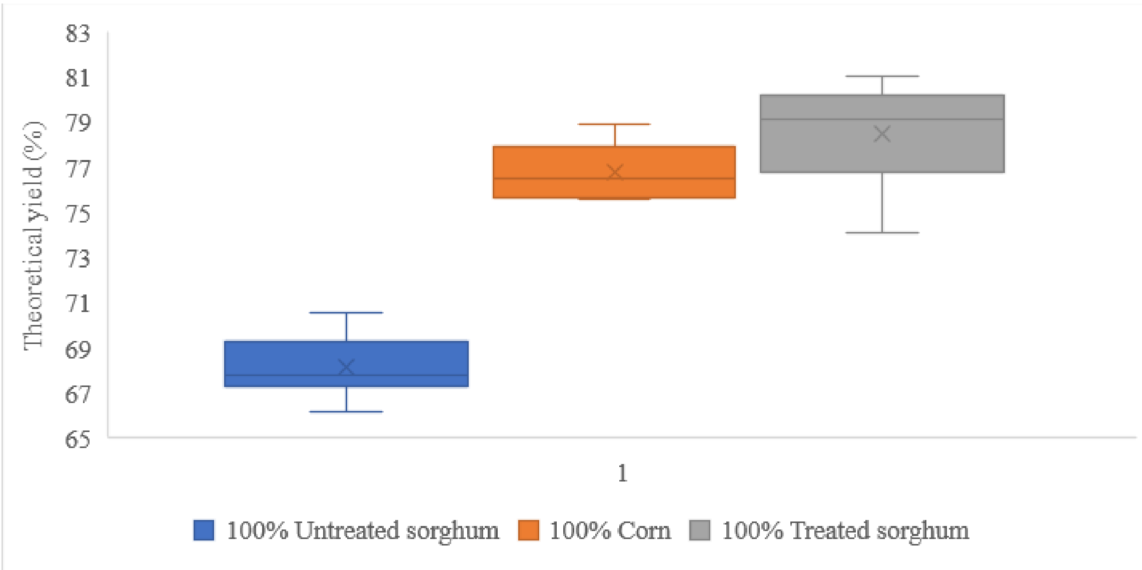


Figure 5. Treated and untreated sorghum theoretical yield comparisons, no-cellullase

The lower ethanol produced when using untreated sorghum, can be related to the tannin content of the grains. The protein binding capabilities of the tannins triggers a lower activity of the enzymes. This is consistent with the observations made by Awika et al. (2004) and Nkomba et al. (2016) regarding tannins interfering with digestive enzymes activity in which amylases were tested among others.

Table 4. Average theoretical yield (%) of sorghum fermentations, no cellulase

Treatment	Expected Ethanol (ml)	Ethanol Produced (ml)	Theoretical yield (%)[7]
100% Corn	12.83	9.85	76.79%
100% Treated sorghum	12.56	9.85	78.47%
100% Untreated sorghum	12.13	8.26	68.15%

It is important to mention that expected ethanol changes due to variations on the starch content of the grains. For further experiments, the changes will be also related to the different amount of starch in corn-treated sorghum mixtures.

This experiment also generated a yield of 394 l/MT for corn, 330.59 l/MT for untreated sorghum and 394.17 l/MT for treated sorghum. All these results are expressed on dry basis. An example of how to calculate the ethanol produced per ton of solids can be seen in Appendix A.

4.2.2. Effect of alkaline tannin removal on fermentation of corn and sorghum mixtures

Different mixtures of corn and treated sorghum were investigated to determine the effect of alkaline pretreatment on ethanol produced and theoretical yield . The mean ethanol values obtained for those fermentations can be seen in Table 5.

[7] Average of six replicates.

Table 5. Ethanol generated by corn and sorghum mixtures, no cellulase

Experiment	Ethanol (% w/v) at 96 h of fermentation time [8]
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

From the data in Table 6, it can be said that the usage of 25, 50 or 75% treated sorghum improved the theoretical yield compared to the usage of 100% corn or 100% untreated sorghum.

Table 6 Theoretical yield (%) for mixtures of corn and treated sorghum, no cellulase

Experiment	Expected Ethanol (ml)	Ethanol Produced (ml)	Theoretical yield (%)[9]
75% Corn + 25% Treated sorghum	127.60	103.78	81.33%
50% Corn + 50% Treated sorghum	126.93	102.05	80.40%
25% Corn + 75% Treated sorghum	126.26	102.35	81.07%

Correspondingly, the ethanol yield calculated as the liters of ethanol produced per metric ton of solids fermented was higher for all three cases: 415.10 l/MT for 25% treated

[8] Average of six replicates.
[9] Average of six replicates.

sorghum, 408.19 l/MT for 50% treated sorghum and 409.40 l/MT for 75% treated sorghum.

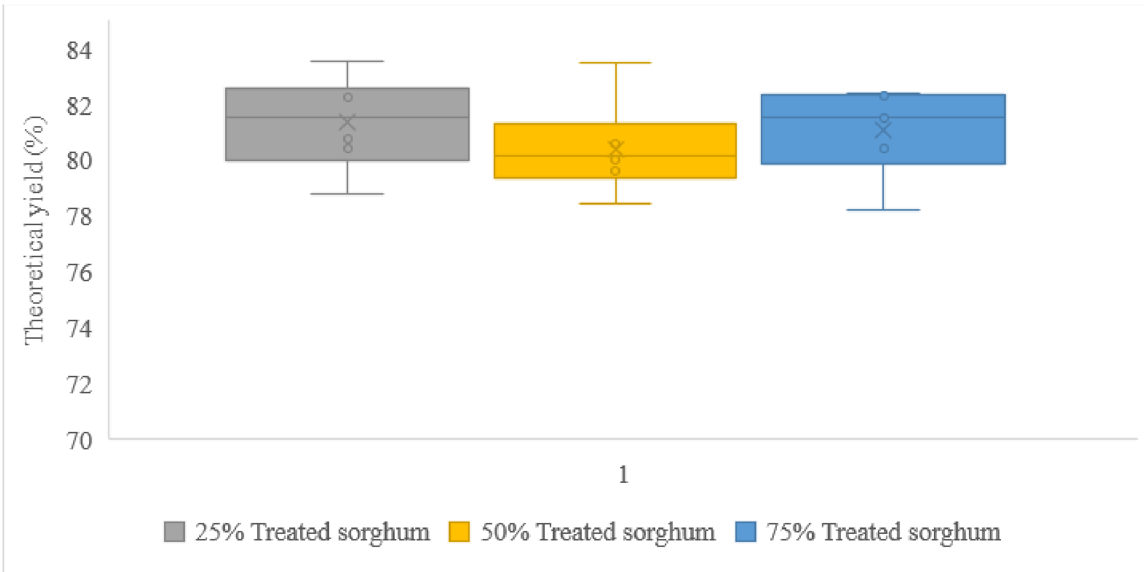


Figure 6. Mixtures of corn and treated sorghum theoretical yield, no cellulase

4.2.3. Overall comparisons

The results of theoretical yield (%) for all the non-cellulase fermentations can be seen in Figure 7. When comparing all fermentations, it can be said that there is at least one mean statistically different from the rest (P <0.0001).

By analyzing Figure 8, the means of the experiments with 25, 50, 75 and 100% treated sorghum are not significantly different from each other. It is important to point out that these experiments produced the highest ethanol theoretical yield . This is an interesting finding because it may help to the economics of an industrial facility. If corn

price increases, the use of alkaline tannin removal will allow the plant to run with up to 100% of treated sorghum in their mashing procedure, making possible the usage of a cheaper feedstock. On the other hand, if corn is cheaper than sorghum, the plant can run at 25% treated sorghum and still have better yields of ethanol compared to the use of 100% corn or 100% untreated sorghum.

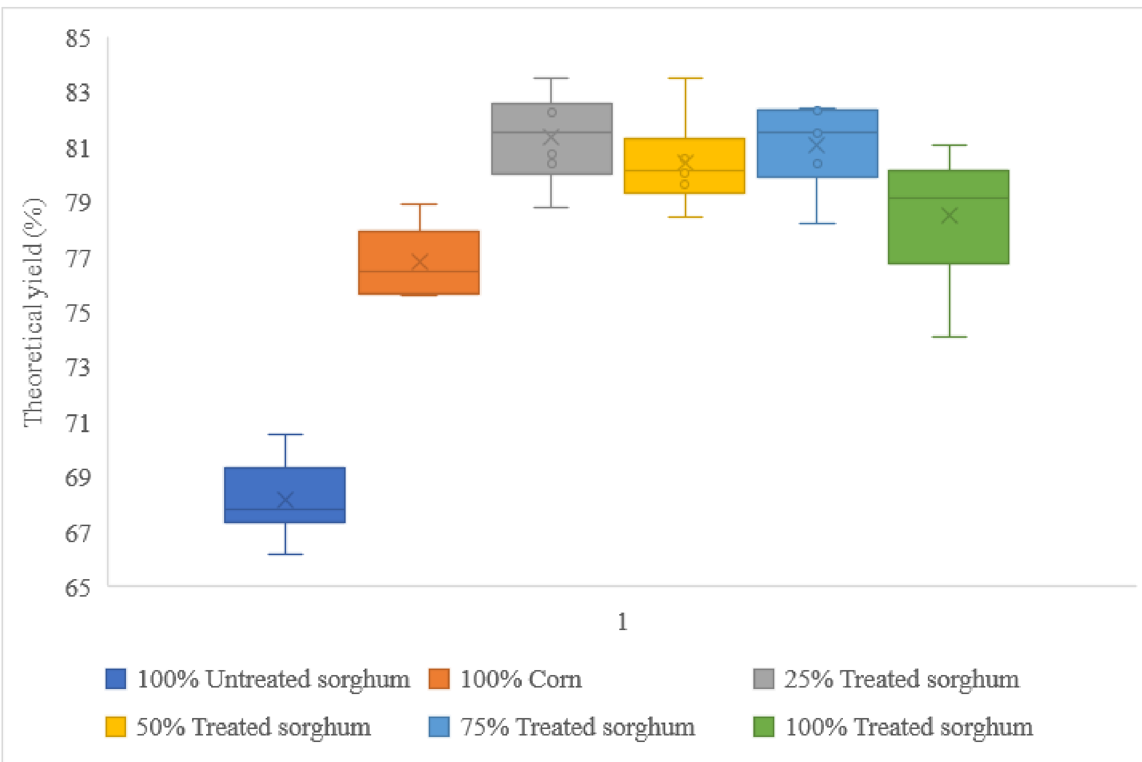


Figure 7. Theoretical yield of non-cellulase fermentations

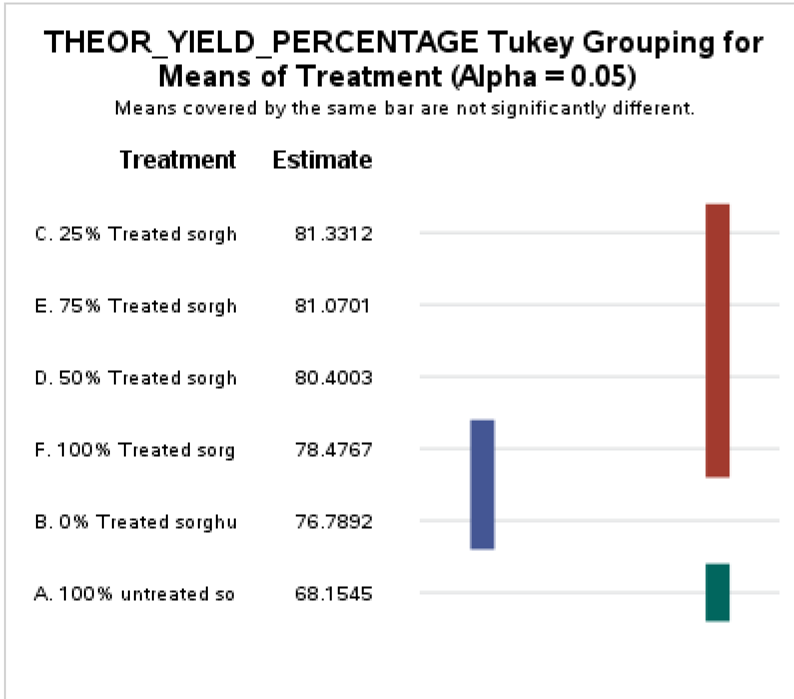


Figure 8. Theoretical yield comparisons for non-cellulase fermentations (HSD tests)

4.3. Cellulase fermentations

4.3.1. Effect of alkaline tannin removal on 100% sorghum fermentation with cellulase addition

For this experiment, the baseline made with corn produced an average of 11.03 ± 0.13 % w/v ethanol after 96 hours of fermentation. The results of fermentation of treated and untreated sorghum with the addition of a cellulase can be seen in Table 7. For this case, ethanol increased from 8.77 ± 0.18 to 11.29 ± 0.21 % w/v.

Table 7. Corn and sorghum fermentations with cellulase

Experiment	**Ethanol (% w/v) at 96 h of fermentation time** [10]
100% Untreated sorghum	8.77 ± 0.18
100% Corn	11.03 ± 0.13
100% Treated sorghum	11.29 ± 0.21

When comparing average theoretical yields (%), the baseline of corn resulted in 89.81 ± 1.16% theoretical yield. Also, 100% untreated sorghum resulted in an ethanol production of 69.77 ± 1.65% of the theoretical whereas for 100% treated sorghum this value was 94.61 ± 1.93%. The trend that follows the theoretical yield as a function of the feedstock used can be seen in Figure 9. This trend is like Figure 5, with the main difference of an increase in the yield obtained in the 100% corn and 100% treated sorghum fermentations.

Similarly to 100% treated and 100% untreated sorghum fermentation without cellulase, the use of the alkaline removal treatment significantly improved the percentage of the theoretical yield achieved in fermentation ($P<0.0001$). The comparison of the three fermentations can be seen in Figure 10. In this case, as a difference with the non-cellulase fermentations, the highest theoretical yield was achieved for 100% treated sorghum.

[10] Average of six replicates.

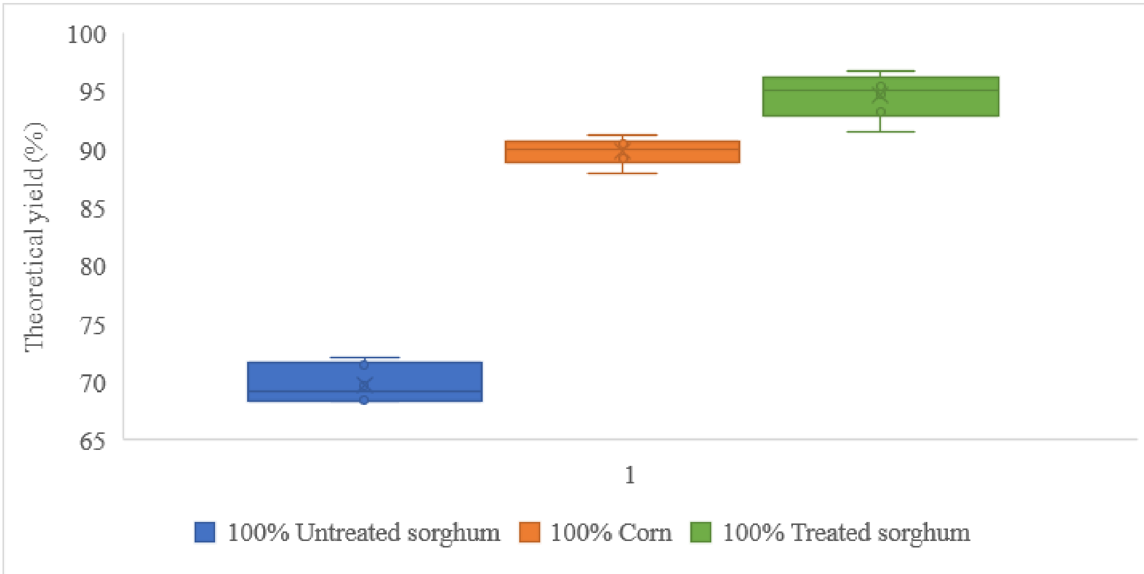


Figure 9. Treated and untreated sorghum average theoretical yield comparisons with cellulase

The use of cellulase helped increase the ethanol produced in all three cases. This may be related to the ability of CTec2® (mixture of cellulase, hemicellulase and beta-glucosidase) to lower the viscosity during liquefaction and fermentation. The lower the viscosity, the higher the activities of enzymes and therefore the better the yields on fermentations.

Table 8. Average theoretical yield of sorghum fermentations with cellulase

Experiment	Expected Ethanol (ml)	Ethanol Produced (ml)	Theoretical yield (%)[11]
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%

Correspondingly, the yield expressed as liters per metric ton of dry solids increased. The values are 364.51 l/MT for 100% untreated sorghum, 486.79 l/MT for 100% treated sorghum and 473.73 l/MT for 100% corn.

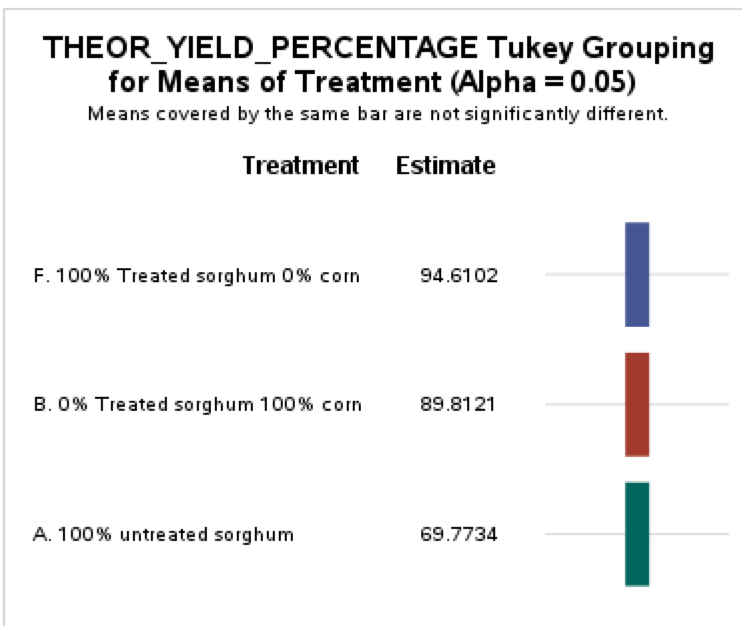


Figure 10. Theoretical yield comparisons (HSD test) of sorghum fermentations with cellulase

[11] Average of six replicates.

4.3.2. Effect of alkaline tannin removal and cellulase on fermentation of corn and sorghum mixtures

The effect of tannin removal and cellulase on ethanol produced by fermentation can be seen in Table 9.

Table 9. Ethanol generated by corn and sorghum mixtures with cellulases

Experiment	Ethanol (% w/v) at 96 h of fermentation time [12]
25% Treated sorghum	10.93 ± 0.22
50% Treated sorghum	11.22 ± 0.15
75% Treated sorghum	11.19 ± 0.19

The theoretical ethanol yield in this experiment also increased when comparing to non cellulase treatment and it can be seen in Table 10. According to Tukey's Studentized Range the mean theoretical yield generated by each experiment are not statistically different for 50 and 75% treated sorghum and those are different from the 25% treated sorghum.

The mean values obtained for theoretical yield are 89.39 ± 1.85% for 25% treated sorghum, 92.76% ± 1.31% for 50% treated sorghum and 93.06 ± 1.57%. These values can also be found in Table 10. Figure 11 shows a box and whisker plot with the results of these fermentations.

[12] Average of six replicates.

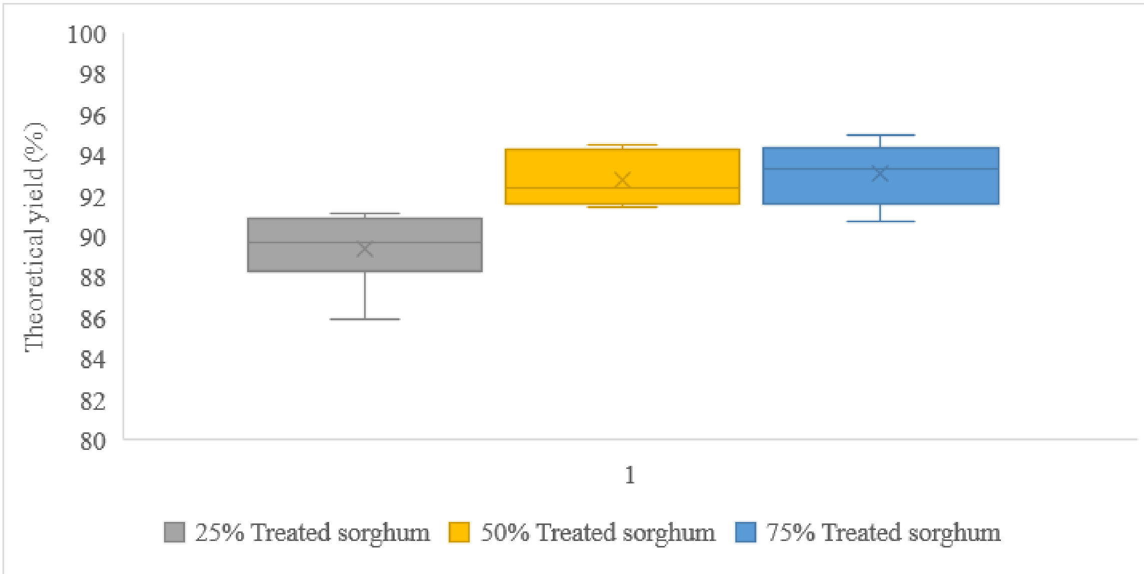


Figure 11. Mixtures of corn and treated sorghum average theoretical yield (%) with cellulase

The yield expressed as liters of ethanol per metric ton of dry solids increased. The values are 468.59 l/MT for 25% treated sorghum, 483.29 l/MT for 50% treated sorghum and 481.81 l/MT for 75% treated sorghum.

Table 10. Theoretical yield for mixtures of corn and treated sorghum with cellulase

Experiment	Expected Ethanol (ml)	Ethanol Produced (ml)	Theoretical yield (%)
75% Corn + 25% Treated sorghum	131.06	117.15	89.38%
50% Corn + 50% Treated sorghum	130.25	120.82	92.76%
25% Corn + 75% Treated sorghum	129.44	120.45	93.05%

4.3.3. Overall comparisons

The results of theoretical yield for all fermentations when using cellulase can be seen in Figure 12. When comparing all fermentations, it can be said there is at least one mean statistically different from the rest ($P < 0.0001$).

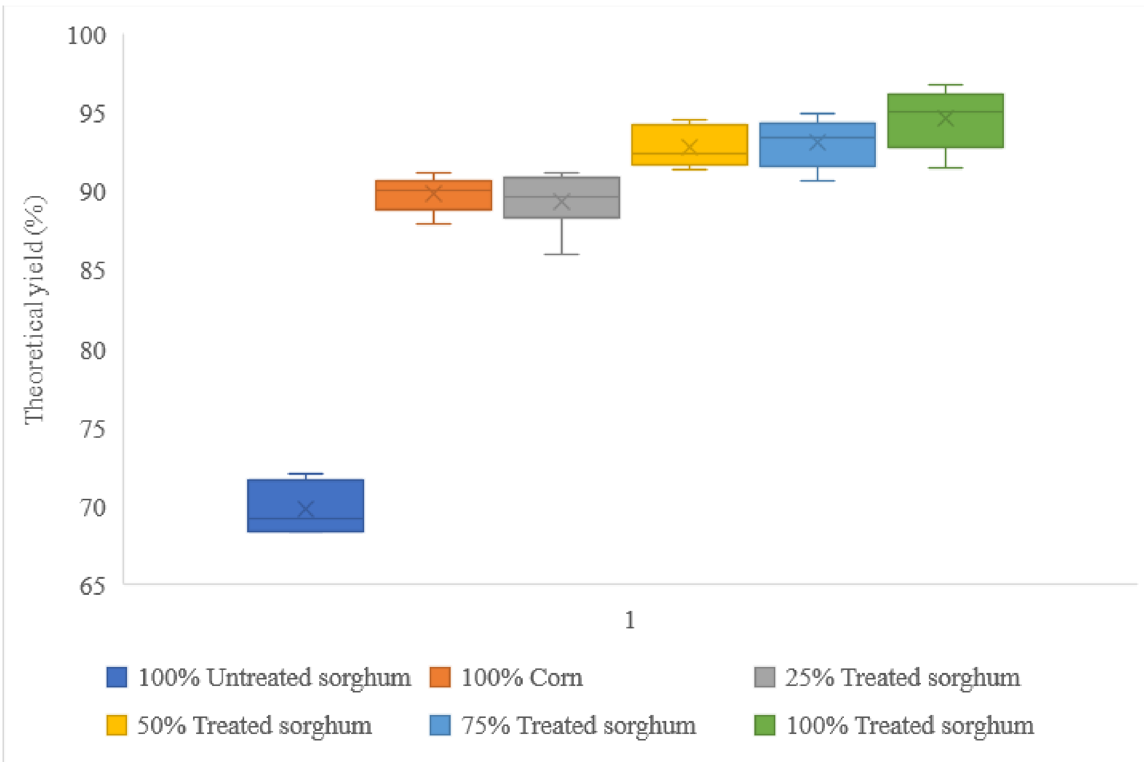


Figure 12. Theoretical yield of fermentations with cellulase

Figure 13 summarizes the results of the comparisons of theoretical yield (%) for all fermentations with cellulase (Tukey's Studentized Range). From an overall analysis it can be said that all cellulase experiments with treated sorghum are higher than the one without cellulases. This may be related to the effectiveness of CTec2® in lowering the viscosity and improving the efficiency of the rest of the enzymes. Also, the alkaline

tannin removal may contribute as a pretreatment for lignin removal, exposing even more cellulose to the activity of CTec® enzymes. This explains the higher ethanol production. Yeast is not only using glucose that comes from starch hydrolysis, but also from cellulose to ferment.

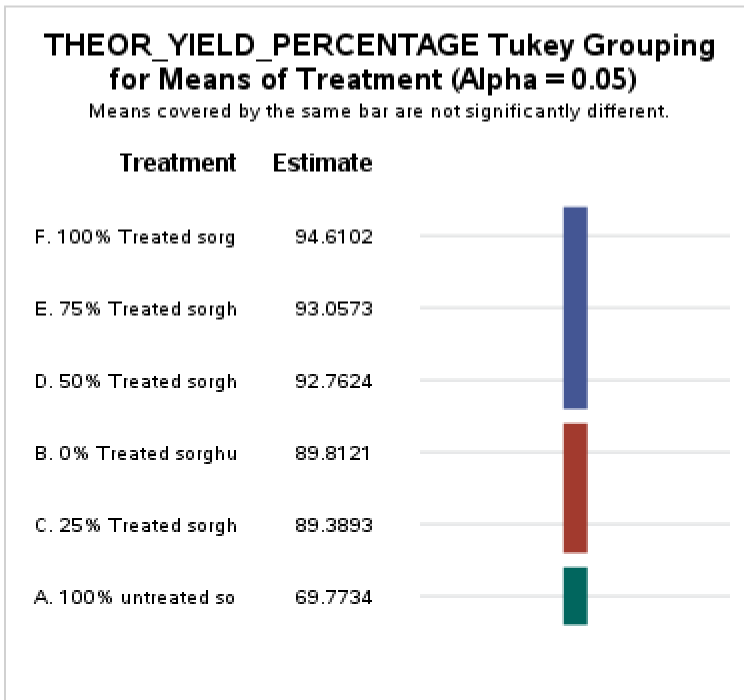


Figure 13. Mean comparisons (HSD test) of all fermentations with cellulase

5. Conclusion

Alkaline tannin removal pretreatment has been effective by removing 87.6% of the tannins of sorghum with only 2.25% loss (dry basis) and a 1.23% increase in starch availability. A downside of the treatment is the higher moisture content that requires further drying to store and mill the grain (30.58%).

The use of the alkaline tannin removal allowed a significant increase on the theoretical yield obtained from fermentations when using 100% sorghum comparable to the corn baseline. The theoretical yield increased from 68.15 ± 1.46% to 78.48 ± 2.47% and the corn baseline is 76.79 ± 1.27%. When considering non-cellulase experiments, the highest ethanol production can be obtained with 25, 50, 75 or 100% treated sorghum in the mix.

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

Overall, tannin removal by alkaline pretreatment is an effective way to deal with tannins when using sorghum to produce bioethanol at a laboratory scale. Further study is recommended to determine the feasibility at a larger scale.

CHAPTER THREE:
CONCLUSIONS AND FUTURE RESEARCH

This is a study on how an alkaline tannin removal affects the ethanol production. Experiments were performed in a laboratory scale (batch, shake-flasks) using *Saccharomyces cerevisiae* as the organism that carries out fermentation. In some of the runs, cellulase was added to evaluate whether alkaline tannin removal worked as a cellulose pretreatment or not.

Sorghum is an interesting feedstock for ethanol production. It has outstanding characteristics that can contribute to make it a suitable alternative to reduce the use of corn utilized to produce biofuel. This can contribute to make bioethanol production a more sustainable process. Also, feedstock diversification will contribute to the economics of the industrial facility. This can help control the cost of the ethanol when reducing the risk of price volatility among multiple feedstocks. The tannin removal process -without the use of cellulase- has achieved the maximum theoretical yield in a range from 25% to 100% sorghum in the solids comprising the mash. For these experiments, the average theoretical yield was 80.3%. An industrial facility can work within this range and adjust the percent sorghum in the mash according to feedstock prices.

In the results obtained it can be seen the potential of alkaline pretreatment for tannin removal. It can remove 87.6% of the tannins of sorghum with 2.25% loss (dry basis) and a 1.23% increase in starch availability. Also, this reduction significantly improved ($P < 0.0001$) ethanol production of sorghum or mixtures with corn.

Cellulase in combination with the use of the alkaline tannin removal generated major improvements in ethanol production compared to the non-cellulase experiments. The highest theoretical yield was achieved when using 50, 75 or 100% treated sorghum. The average value of theoretical yield for those experiments was 93.5%.

When analyzing a biorefinery stand point, alternatives are needed to determine the possible use of the by-products obtained in this process. The fate of carbon dioxide and wet distillers grains with solubles (or dry distillers grains with solubles) is well known in this industry. This comprises carbon dioxide recovery and purification for further uses and utilization of WDGS or DDGS for animal feed as the most common ones. The key difference from this biorefinery to a traditional ethanol plant is the tannin solution by-product. This is a highly alkaline product containing tannins. Research must be conducted to determine the best way to recover and purify those tannins. Possible uses may include use as mordants in dyes and, due to its protein binding ability, clarifier and antifoam agent in beer and wine industry. Regarding the sodium hydroxide solution remaining, it could be reused in the process to decrease the need of fresh sodium hydroxide or used as a carbon sequestration agent.

When considering future research options, additional ideas may include the evaluation of this process at larger-scale fermentations (larger laboratory-scale volumes or pilot plant scale), optimization of the alkaline tannin removal process to minimize water consumption and decrease sodium hydroxide use and finally a techno-economic analysis to determine the feasibility of the use of this process in an industrial scale.

APPENDICES

APPENDIX A: Theoretical ethanol calculations

A.1. Calculation of theoretical ethanol production from starch

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

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

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

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

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

The theoretical ethanol production can be calculated from the fermentation stoichiometry:

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

Water consumption during this process can be calculated as:

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

The final water volume in the flask is:

$$75ml - 1.96ml = 73.04ml$$

Ethanol production will produce a volume increase such as:

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

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

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

$$V_F = 8.1577 \times V_{EtOH}$$

Substituting,

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

$$V_{EtOH} = 10.20ml$$

The mass of ethanol can be calculated as:

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

Finally, the fermentation efficiency is:

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

Calculation of ethanol per ton

$$\frac{10.20ml}{25gDS} \times 1000000 \frac{g}{ton} \times \frac{1}{1000} \frac{l}{ml} = 408 \frac{l}{ton}$$

A.2. Calculation of theoretical ethanol production from starch and cellulose

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

Considering the starch content of corn 71.4% and treated sorghum 69.9%. Also, cellulose content in corn 2% and 1.7% in treated sorghum, all values in dry basis, and assuming a 50% corn + 50% sorghum mixture, the total of fermentable sugars are calculated as:

$$25g \times \left[(50\% \times 73.4\%) + (50\% \times 71.6\%) \right] = 18.13g$$

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

$$18.13g \times 1.111 = 20.14g$$

The theoretical ethanol production can be calculated from the fermentation stoichiometry:

$$20.14g \times 0.511 = 10.29g = 13.03ml$$

Water consumption during this process can be calculated as:

$$18.13g \times 0.111 = 2.01g = 2.01ml$$

The final water volume in the flask is:

$$75ml - 2.01ml = 72.99ml$$

Ethanol production will produce a volume increase such as:

$$V_F = 72.99 + V_{EtOH}$$

The HPLC ethanol concentration, for this example 11.22% w/v. Then:

$$\frac{0.79 \times V_{EtOH}}{V_F} = 0.1122$$
$$V_F = 7.04 \times V_{EtOH}$$

Substituting,

$$7.04 \times V_{EtOH} = 72.99 + V_{EtOH}$$
$$V_{EtOH} = 12.08ml$$

The mass of ethanol can be calculated as:

$$12.08ml \times 0.79 \frac{g}{ml} = 9.54g$$

Finally, the fermentation efficiency is:

$$\frac{9.54g}{10.29g} \times 100 = 92.71\%$$

Calculation of ethanol per ton

$$\frac{12.08ml}{25gDS} \times 1000000 \frac{g}{ton} \times \frac{1}{1000} \frac{l}{ml} = 483.2 \frac{l}{ton}$$

APPENDIX B: Weight loss track

100% Untreated sorghum – Without cellulase

Sampling times	Relative time
4/1/2018 12:00	0 hours
4/2/2018 12:00	24 hours
4/2/2018 20:00	32 hours
4/3/2018 11:00	47 hours
4/3/2018 18:30	54 hours
4/4/2018 12:00	72 hours
4/4/2018 18:00	78 hours
4/5/2018 12:00	96 hours

Weight loss

Sample ID	0 hours	24 hours	32 hours	47 hours	54 hours	72 hours	78 hours	96 hours
13	166.6154	162.7368	161.7262	160.4827	159.968	159.093	158.913	158.681
14	169.4315	165.6475	164.6479	163.4167	162.905	162.017	161.837	161.609
15	178.9089	175.1106	174.0725	172.8164	172.301	171.369	171.116	170.805
16	169.7003	165.8303	164.7849	163.5083	162.976	162.023	161.797	161.545
17	168.506	164.8454	163.8375	162.5806	162.06	161.115	160.846	160.494
18	173.3936	169.8346	168.8575	167.6384	167.13	166.235	165.972	165.486

Carbon dioxide production

Time	0 hours	24 hours	32 hours	47 hours	54 hours	72 hours	78 hours	96 hours
13	0	3.8786	4.8892	6.1327	6.6474	7.5221	7.7026	7.9347
14	0	3.784	4.7836	6.0148	6.5264	7.4145	7.5941	7.8225
15	0	3.7983	4.8364	6.0925	6.6075	7.5399	7.7929	8.1039
16	0	3.87	4.9154	6.192	6.7245	7.677	7.9035	8.1549
17	0	3.6606	4.6685	5.9254	6.4461	7.3906	7.66	8.0124
18	0	3.559	4.5361	5.7552	6.2636	7.1584	7.4215	7.9073
CO2 No-Cellulase	0	3.8203	4.8364	6.08	6.59377	7.49217	7.69653	7.9537
Change in CO2		3.8203	1.0161	1.2436	0.51377	0.8984	0.20437	0.25717
Change in time		24	8	15	7.5	17.5	6	18
Change in 8 hours		1.273433	1.0161	0.66325	0.54802	0.4107	0.27249	0.1143
Percentage change		#DIV/0!	26.60%	13.71%	9.01%	6.23%	3.64%	1.49%

100% Untreated sorghum – With cellulase

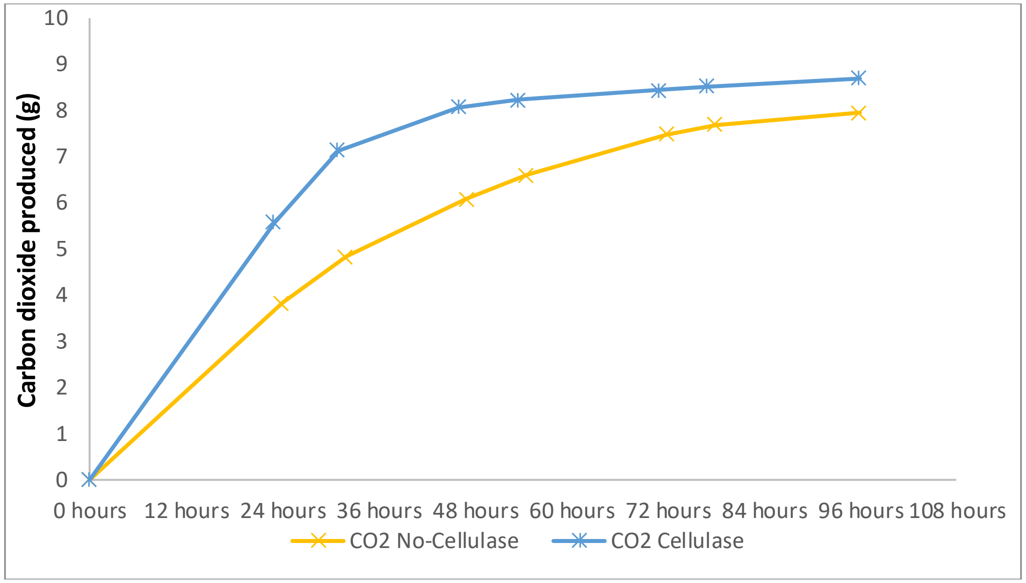
Sampling times	Relative time
4/1/2018 13:00	0 hours
4/2/2018 12:00	23 hours
4/2/2018 20:00	31 hours
4/3/2018 11:00	46 hours
4/3/2018 18:30	53 hours
4/4/2018 12:00	71 hours
4/4/2018 18:00	77 hours
4/5/2018 13:00	96 hours

Weight loss

Sample ID	0 hours	23 hours	31 hours	46 hours	53 hours	71 hours	77 hours	96 hours
19	165.4722	159.848	158.3632	157.479	157.35	157.152	157.048	156.922
20	176.7528	171.0483	169.4839	168.5597	168.426	168.209	168.14	167.962
21	177.7737	172.1954	170.6022	169.6617	169.513	169.285	169.235	169.02
22	173.481	167.8738	166.2852	165.3229	165.178	164.955	164.884	164.708
23	170.1963	164.5263	162.9609	161.9978	161.857	161.635	161.562	161.383
24	164.4006	159.0747	157.5285	156.569	156.417	156.184	156.114	155.933

Carbon dioxide production

Time	0 hours	23 hours	31 hours	46 hours	53 hours	71 hours	77 hours	96 hours
19	0	5.6242	7.109	7.9932	8.1221	8.32	8.424	8.5502
20	0	5.7045	7.2689	8.1931	8.3264	8.5436	8.6127	8.7904
21	0	5.5783	7.1715	8.112	8.261	8.4888	8.5392	8.7541
22	0	5.6072	7.1958	8.1581	8.3031	8.526	8.5974	8.773
23	0	5.67	7.2354	8.1985	8.3393	8.5615	8.6343	8.8135
24	0	5.3259	6.8721	7.8316	7.9838	8.2162	8.2867	8.4674
CO2 Cellulase	0	5.58501	7.1421	8.08108	8.2226	8.4426	8.5157	8.6914
Change in CO2		5.58501	1.5571	0.93896	0.1415	0.2200	0.0730	0.1757
Change in time		23	8	15	7.5	17.5	6	19
Change in 8 hours		1.942614	1.5571	0.50078	0.1509	0.1006	0.0973	0.0739
Percentage change		#DIV/0!	27.88%	7.01%	1.87%	1.22%	1.15%	0.87%



100% Corn – Without cellulase

Sampling times	Relative time
3/31/2018 12:00	0 hours
3/31/2018 19:00	7 hours
4/1/2018 9:30	21 hours
4/1/2018 13:30	25 hours
4/2/2018 12:00	48 hours
4/2/2018 20:00	56 hours
4/3/2018 11:00	71 hours
4/3/2018 18:30	78 hours
4/4/2018 12:00	96 hours

Weight loss

Sample ID	0 hours	7 hours	21 hours	25 hours	48 hours	56 hours	71 hours	78 hours	96 hours
1	176.4924	176.2164	172.8194	172.2518	170.014	169.408	168.585	168.252	167.6311
2	169.6847	169.4029	166.0053	165.4457	163.263	162.688	161.919	161.575	160.9675
3	169.2249	168.9664	165.4848	164.9091	162.664	162.075	161.283	160.935	160.327
4	175.1686	174.9124	171.347	170.763	168.474	167.88	167.067	166.717	166.0923
5	167.3575	167.105	163.5909	163.0068	160.733	160.138	159.333	158.988	158.3612
6	172.9689	172.6696	169.2684	168.706	166.458	165.872	165.079	164.743	164.1697

Carbon dioxide production

Sample ID	0 hours	7 hours	21 hours	25 hours	48 hours	56 hours	71 hours	78 hours	96 hours
1	0	0.276	3.673	4.2406	6.4784	7.0849	7.9074	8.2404	8.8613
2	0	0.2818	3.6794	4.239	6.4215	6.9967	7.7657	8.1098	8.7172
3	0	0.2585	3.7401	4.3158	6.5611	7.1504	7.942	8.2903	8.8979
4	0	0.2562	3.8216	4.4056	6.6948	7.2888	8.1019	8.4512	9.0763
5	0	0.2525	3.7666	4.3507	6.6243	7.2198	8.0243	8.37	8.9963
6	0	0.2993	3.7005	4.2629	6.5105	7.0969	7.8902	8.2255	8.7992
Average CO2 production	0	0.2721	3.6975	4.2651	6.487	7.07733	7.8717	8.2135	8.8254
Change in CO2		0.2721	3.4254	0.5676333	2.22187	0.59033	0.79437	0.3418	0.6119
Change in time		7	14.5	4	22.5	8	15	7.5	17.5
Change in 8 hours		0.31097	1.8898	1.1352	0.79	0.59033	0.42366	0.36459	0.27975
Percentage change		#DIV/0!	694.55%	30.70%	18.52%	9.10%	5.99%	4.63%	3.41%

100% Corn – With cellulase

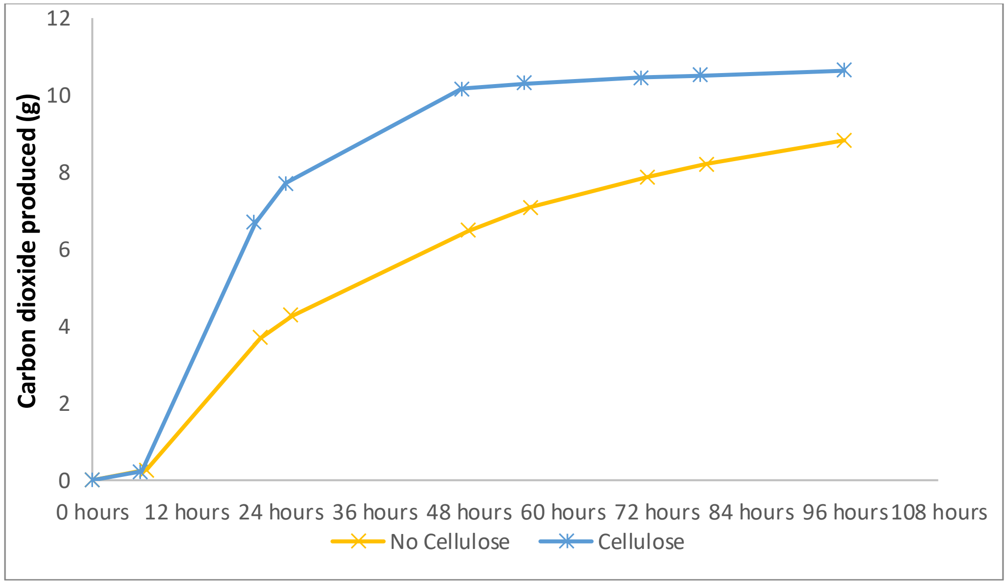
Sampling times	Relative time
3/31/2018 12:45	0 hours
3/31/2018 19:00	6 hours
4/1/2018 9:30	20 hours
4/1/2018 13:30	24 hours
4/2/2018 12:00	47 hours
4/2/2018 20:00	55 hours
4/3/2018 11:00	70 hours
4/3/2018 18:30	77 hours
4/4/2018 12:45	96 hours

Weight loss

Sample ID	0 hours	6 hours	20 hours	24 hours	47 hours	55 hours	70 hours	77 hours	96 hours
7	167.5075	167.2607	160.8977	159.8931	157.431	157.295	157.136	157.07	156.9499
8	174.145	173.9182	167.5215	166.5167	164.041	163.91	163.753	163.691	163.5716
9	175.0782	174.8425	168.3113	167.2895	164.84	164.709	164.559	164.498	164.3778
10	173.446	173.2236	166.7929	165.7745	163.304	163.167	163.013	162.951	162.8331
11	177.4618	177.2397	170.7641	169.7395	167.257	167.125	166.975	166.913	166.797
12	176.6713	176.4447	169.8991	168.8582	166.434	166.307	166.159	166.1	165.986

Carbon dioxide production

Time	0 hours	6 hours	20 hours	24 hours	47 hours	55 hours	70 hours	77 hours	96 hours
7	0	0.2468	6.6098	7.6144	10.0769	10.2125	10.3713	10.4379	10.5576
8	0	0.2268	6.6235	7.6283	10.1038	10.2355	10.3916	10.454	10.5734
9	0	0.2357	6.7669	7.7887	10.2385	10.3691	10.5194	10.58	10.7004
10	0	0.2224	6.6531	7.6715	10.1424	10.2793	10.4328	10.4948	10.6129
11	0	0.2221	6.6977	7.7223	10.2049	10.3365	10.487	10.5489	10.6648
12	0	0.2266	6.7722	7.8131	10.2374	10.3648	10.5126	10.5718	10.6853
Average CO2 production	0	0.23006	6.6872	7.7063	10.1673	10.2996	10.4525	10.5146	10.6324
Change in CO2		0.23006	6.4571	1.0191	2.4609	0.1323	0.15283	0.06212	0.1178
Change in time		6.25	14.5	4	22.5	8	15	7.5	18.25
Change in 8 hours		0.29448	3.5625	2.0383	0.875	0.1323	0.08151	0.06626	0.05165
Percentage change		#DIV/0!	1548.49%	30.48%	11.35%	1.30%	0.79%	0.63%	0.49%



25% Treated sorghum + 75% Corn – Without cellulase

Sampling times	Relative time
4/5/2018 10:45	0 hours
4/6/2018 7:30	20 hours
4/6/2018 20:00	33 hours
4/7/2018 11:30	48 hours
4/8/2018 11:30	72 hours
4/8/2018 18:30	79 hours
4/9/2018 10:45	96 hours

Weight loss

Sample ID	0 hours	20 hours	33 hours	48 hours	72 hours	79 hours	96 hours
1	175.1566	171.2272	169.4242	167.9545	166.564	166.256	165.758
2	171.1745	167.3145	165.5289	164.098	162.754	162.465	161.987
3	169.7315	165.721	163.8522	162.3621	160.941	160.644	160.218
4	176.9608	173.0647	171.2227	169.72	168.319	168.019	167.581
5	172.8301	168.9147	167.1253	165.6775	164.31	164.018	163.516
6	175.3548	171.4228	169.5456	168.0404	166.624	166.342	165.918

Carbon dioxide production

Time	0 hours	20 hours	33 hours	48 hours	72 hours	79 hours	96 hours
1	0	3.9294	5.7324	7.2021	8.5926	8.9009	9.3987
2	0	3.86	5.6456	7.0765	8.4205	8.71	9.1875
3	0	4.0105	5.8793	7.3694	8.7905	9.0871	9.5135
4	0	3.8961	5.7381	7.2408	8.6422	8.9423	9.3803
5	0	3.9154	5.7048	7.1526	8.5199	8.8117	9.3139
6	0	3.932	5.8092	7.3144	8.7311	9.0128	9.4365
CO2 No-Cellulase	0	3.9333	5.7524333	7.216	8.6012	8.89933	9.36657
Change in CO2		3.9333	1.8191333	1.4635667	1.3852	0.29813	0.46723
Change in time		20.75	12.5	15.5	24	7	16.25
Change in 8 hours		1.516453012	1.1642453	0.7553892	0.46173	0.34072	0.23002
Percentage change		#DIV/0!	29.60%	13.13%	6.40%	3.96%	2.58%

25% Treated sorghum + 75% Corn – With cellulase

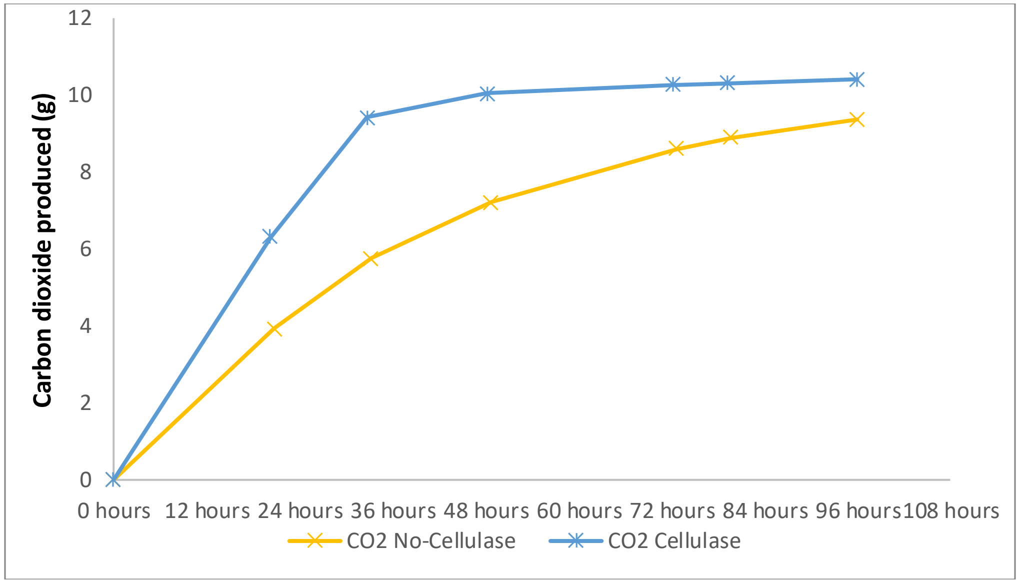
Sampling times	Relative time
4/5/2018 11:15	0 hours
4/6/2018 7:30	20 hours
4/6/2018 20:00	32 hours
4/7/2018 11:30	48 hours
4/8/2018 11:30	72 hours
4/8/2018 18:30	79 hours
4/9/2018 11:15	96 hours

Weight loss

Sample ID	0 hours	20 hours	32 hours	48 hours	72 hours	79 hours	96 hours
7	165.5517	159.2526	156.213	155.6437	155.428	155.376	155.277
8	168.6424	162.3911	159.3013	158.6756	158.452	158.4	158.307
9	174.6246	168.3109	165.1618	164.523	164.307	164.254	164.158
10	171.9394	165.6098	162.5279	161.947	161.735	161.689	161.604
11	177.7052	171.3535	168.2474	167.563	167.343	167.299	167.214
12	175.774	169.4186	166.2382	165.5873	165.359	165.307	165.208

Carbon dioxide production

Time	0 hours	20 hours	32 hours	48 hours	72 hours	79 hours	96 hours
7	0	6.2991	9.3387	9.908	10.1238	10.1756	10.2752
8	0	6.2513	9.3411	9.9668	10.1908	10.2427	10.3353
9	0	6.3137	9.4628	10.1016	10.3179	10.3703	10.4662
10	0	6.3296	9.4115	9.9924	10.2041	10.2507	10.3355
11	0	6.3517	9.4578	10.1422	10.3625	10.4067	10.4912
12	0	6.3554	9.5358	10.1867	10.4147	10.4668	10.5659
CO2 Cellulase	0	6.3168	9.4246167	10.049617	10.269	10.3188	10.4116
Change in CO2		6.3168	3.1078167	0.625	0.21935	0.04983	0.09275
Change in time		20.25	12.5	15.5	24	7	16.75
Change in 8 hours		2.495525926	1.9890027	0.3225806	0.07312	0.05695	0.0443
Percentage change		#DIV/0!	31.49%	3.42%	0.73%	0.55%	0.43%



50% Treated sorghum + 50% Corn – Without cellulase

4/6/2018 10:45	0 hours
4/6/2018 20:00	9 hours
4/7/2018 11:30	24 hours
4/8/2018 11:30	48 hours
4/8/2018 18:30	55 hours
4/9/2018 11:00	72 hours
4/10/2018 10:45	96 hours

Weight loss

Sample ID	0 hours	9 hours	24 hours	48 hours	55 hours	72 hours	96 hours
25	182.8802	182.3775	178.6064	176.0628	175.566	174.677	173.946
26	185.0478	184.5626	180.5832	177.9559	177.449	176.529	175.924
27	191.1718	190.6815	186.7668	184.1356	183.633	182.723	182.095
28	187.669	187.1853	183.2552	180.6575	180.145	179.236	178.598
29	183.187	182.7287	178.8134	176.215	175.706	174.791	174.079
30	184.4696	184.0346	179.9983	177.3429	176.816	175.88	175.236

Carbon dioxide production

Time	0 hours	9 hours	24 hours	48 hours	55 hours	72 hours	96 hours
25	0	0.5027	4.2738	6.8174	7.3146	8.2036	8.934
26	0	0.4852	4.4646	7.0919	7.5988	8.5191	9.1242
27	0	0.4903	4.405	7.0362	7.539	8.4488	9.077
28	0	0.4837	4.4138	7.0115	7.5236	8.4332	9.071
29	0	0.4583	4.3736	6.972	7.4808	8.3964	9.1077
30	0	0.435	4.4713	7.1267	7.6534	8.5894	9.2333
CO2 No-Cellulase	0	0.492733333	4.3811333	6.9818333	7.48413	8.3905	9.04507
Change in CO2		0.492733333	3.8884	2.6007	0.5023	0.90637	0.65457
Change in time		9.25	15.5	24	7	16.5	23.75
Change in 8 hours		0.426147748	2.0069161	0.8669	0.57406	0.43945	0.22049
Percentage change		#DIV/0!	407.30%	19.79%	8.22%	5.87%	2.63%

50% Treated sorghum + 50% Corn – With cellulase

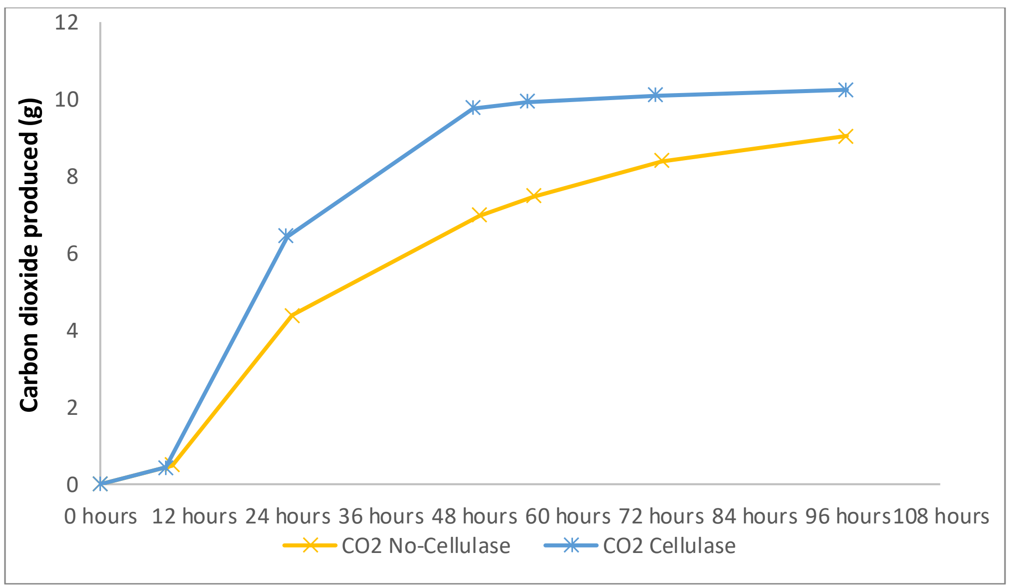
Sampling times	Relative time
4/6/2018 11:30	0 hours
4/6/2018 20:00	8 hours
4/7/2018 11:30	24 hours
4/8/2018 11:30	48 hours
4/8/2018 18:30	55 hours
4/9/2018 11:00	71 hours
4/10/2018 11:30	96 hours

Weight loss

Sample ID	0 hours	8 hours	24 hours	48 hours	55 hours	71 hours	96 hours
31	185.6862	185.2362	179.1479	175.7508	175.592	175.424	175.272
32	188.889	188.4419	182.448	179.1086	178.936	178.773	178.63
33	192.3622	191.9498	185.9118	182.5587	182.386	182.203	182.041
34	187.3382	186.8625	180.8581	177.5496	177.39	177.24	177.09
35	180.9626	180.5637	174.6194	171.325	171.175	171.017	170.879
36	191.8808	191.472	185.4912	182.1925	182.061	181.89	181.75

Carbon dioxide production

Time	0 hours	8 hours	24 hours	48 hours	55 hours	71 hours	96 hours
31	0	0.45	6.5383	9.9354	10.0938	10.2622	10.414
32	0	0.4471	6.441	9.7804	9.953	10.1164	10.2588
33	0	0.4124	6.4504	9.8035	9.9764	10.1593	10.3211
34	0	0.4757	6.4801	9.7886	9.9481	10.0983	10.2487
35	0	0.3989	6.3432	9.6376	9.7876	9.9456	10.0834
36	0	0.4088	6.3896	9.6883	9.8203	9.9909	10.1304
CO2 Cellulase	0	0.43215	6.4404333	9.7723	9.92987	10.0955	10.2427
Change in CO2		0.43215	6.0082833	3.3318667	0.15757	0.16558	0.14728
Change in time		8.5	15.5	24	7	16.5	24.5
Change in 8 hours		0.406729412	3.1010495	1.1106222	0.18008	0.08028	0.04809
Percentage change		#DIV/0!	717.59%	17.24%	1.84%	0.81%	0.48%



75% Treated sorghum + 25% Corn – Without cellulase

Sampling times	Relative time
4/18/2018 11:00	0 hours
4/19/2018 12:00	25 hours
4/19/2018 18:30	31 hours
4/20/2018 11:00	48 hours
4/21/2018 17:00	78 hours
4/22/2018 11:00	96 hours

Weight loss

Sample ID	0 hours	25 hours	31 hours	48 hours	78 hours	96 hours
1	169.0027	164.5352	163.612	162.0899	160.437	159.811
2	166.6624	162.2289	161.3293	159.8227	158.17	157.537
3	164.3165	159.9519	159.0608	157.564	155.929	155.316
4	171.368	166.9678	166.0628	164.5628	162.932	162.308
5	177.264	172.7824	171.8514	170.2918	168.572	167.93
6	166.4599	162.0685	161.1604	159.6453	157.986	157.365

Carbon dioxide production

Time	0 hours	25 hours	31 hours	48 hours	78 hours	96 hours
1	0	4.4675	5.3907	6.9128	8.5659	9.1921
2	0	4.4335	5.3331	6.8397	8.4929	9.1254
3	0	4.3646	5.2557	6.7525	8.3875	9.0003
4	0	4.4002	5.3052	6.8052	8.4362	9.0599
5	0	4.4816	5.4126	6.9722	8.6921	9.3336
6	0	4.3914	5.2995	6.8146	8.4743	9.0954
CO2 No-Cellulase	0	4.421866667	5.3265	6.835	8.4821	9.10593
Change in CO2		4.421866667	0.9046333	1.5085	1.6471	0.62383
Change in time		25	6.5	16.5	30	18
Change in 8 hours		1.414997333	1.1133949	0.7313939	0.43923	0.27726
Percentage change		#DIV/0!	25.18%	13.73%	6.43%	3.27%

75% Treated sorghum + 25% Corn – With cellulase

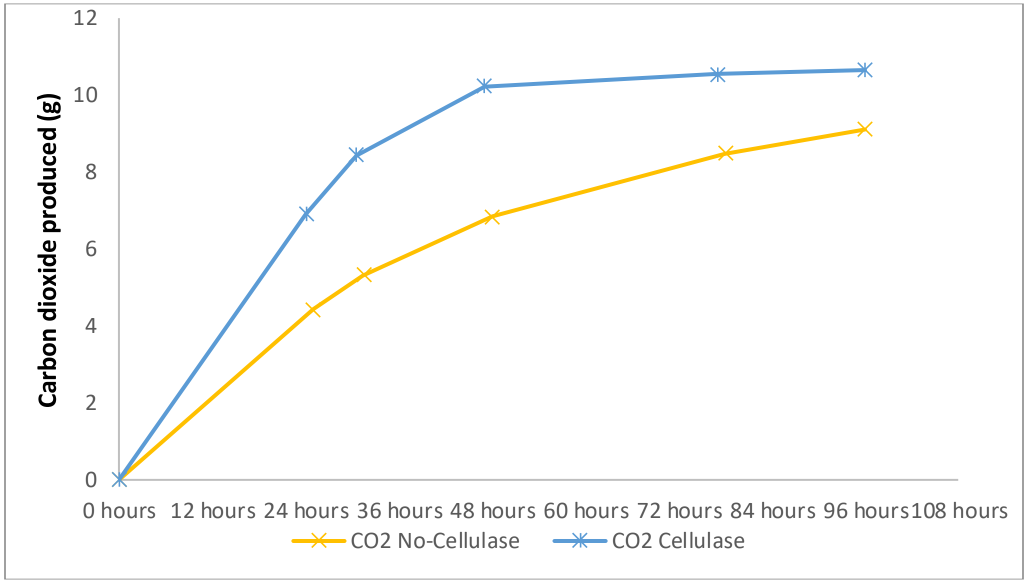
Sampling times	Relative time
4/18/2018 12:00	0 hours
4/19/2018 12:00	24 hours
4/19/2018 18:30	30 hours
4/20/2018 11:00	47 hours
4/21/2018 17:00	77 hours
4/22/2018 12:00	96 hours

Weight loss

Sample ID	0 hours	24 hours	30 hours	47 hours	77 hours	96 hours
7	168.0328	161.2009	159.6728	157.8376	157.519	157.413
8	173.364	166.6177	165.0967	163.226	162.913	162.805
9	167.0698	160.1514	158.605	156.8523	156.548	156.443
10	177.7537	170.6818	169.1018	167.3661	167.059	166.961
11	165.0539	158.243	156.7222	155.0014	154.702	154.599
12	178.8014	171.8275	170.2315	168.3597	168.047	167.951

Carbon dioxide production

Time	0 hours	24 hours	30 hours	47 hours	77 hours	96 hours
7	0	6.8319	8.36	10.1952	10.5138	10.6198
8	0	6.7463	8.2673	10.138	10.4515	10.559
9	0	6.9184	8.4648	10.2175	10.522	10.6267
10	0	7.0719	8.6519	10.3876	10.6947	10.7932
11	0	6.8109	8.3317	10.0525	10.3516	10.4546
12	0	6.9739	8.5699	10.4417	10.7546	10.8506
CO2 Cellulase	0	6.892216667	8.4409333	10.23875	10.548	10.6507
Change in CO2		6.892216667	1.5487167	1.7978167	0.30928	0.10262
Change in time		24	6.5	16.5	30	19
Change in 8 hours		2.297405556	1.9061128	0.8716687	0.08248	0.04321
Percentage change		#DIV/0!	27.66%	10.33%	0.81%	0.41%



100% Treated sorghum – Without cellulase

Sampling times	Relative time
4/18/2018 16:00	0 hours
4/19/2018 12:00	20 hours
4/19/2018 18:30	26 hours
4/20/2018 11:00	43 hours
4/21/2018 17:00	73 hours
4/22/2018 16:00	96 hours

Weight loss

Sample ID	0 hours	20 hours	26 hours	43 hours	73 hours	96 hours
25	186.6467	183.2565	182.0963	180.346	178.475	177.766
26	186.579	183.2199	182.065	180.336	178.48	177.789
27	183.8258	180.34	179.1507	177.3533	175.493	174.783
28	189.574	186.1375	184.9618	183.1983	181.335	180.641
29	190.537	187.009	185.8002	183.9735	182.057	181.332
30	188.7523	185.2791	184.1108	182.3517	180.502	179.801

Carbon dioxide production

Time	0 hours	20 hours	26 hours	43 hours	73 hours	96 hours
25	0	3.3902	4.5504	6.3007	8.1715	8.8812
26	0	3.3591	4.514	6.243	8.0988	8.7897
27	0	3.4858	4.6751	6.4725	8.3331	9.0432
28	0	3.4365	4.6122	6.3757	8.2386	8.9335
29	0	3.528	4.7368	6.5635	8.4801	9.205
30	0	3.4732	4.6415	6.4006	8.2499	8.9509
CO2 No-Cellulase	0	3.4117	4.5798333	6.3387333	8.20113	8.9047
Change in CO2		3.4117	1.1681333	1.7589	1.8624	0.70357
Change in time		20	6.5	16.5	30	23
Change in 8 hours		1.36468	1.4377026	0.8528	0.49664	0.24472
Percentage change		#DIV/0!	42.14%	18.62%	7.84%	2.98%

100% Treated sorghum – With cellulase

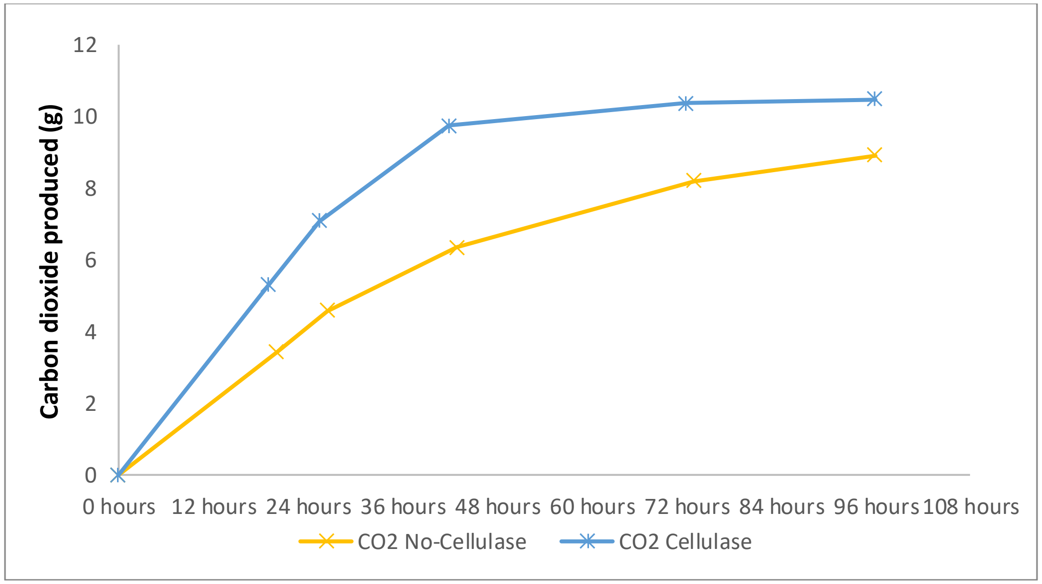
Sampling times	Relative time
4/18/2018 17:00	0 hours
4/19/2018 12:00	19 hours
4/19/2018 18:30	25 hours
4/20/2018 11:00	42 hours
4/21/2018 17:00	72 hours
4/22/2018 17:00	96 hours

Weight loss

Sample ID	0 hours	19 hours	25 hours	42 hours	72 hours	96 hours
31	185.6426	180.2802	178.492	175.7524	175.144	175.042
32	189.0966	183.8176	182.0445	179.386	178.746	178.65
33	190.2084	184.904	183.1506	180.54	179.929	179.826
34	189.0508	183.7674	182.0176	179.3836	178.771	178.67
35	190.0204	184.66	182.878	180.2126	179.552	179.449
36	192.442	187.2037	185.4396	182.7526	182.124	182.02

Carbon dioxide production

Time/sample ID	0 hours	19 hours	25 hours	42 hours	72 hours	96 hours
31	0	5.3624	7.1506	9.8902	10.4985	10.6006
32	0	5.279	7.0521	9.7106	10.3508	10.4464
33	0	5.3044	7.0578	9.6684	10.2792	10.3827
34	0	5.2834	7.0332	9.6672	10.2802	10.3804
35	0	5.3604	7.1424	9.8078	10.4684	10.5712
36	0	5.2383	7.0024	9.6894	10.3182	10.4216
CO2 Cellulase	0	5.30465	7.0730833	9.7389333	10.3659	10.4672
Change in CO2		5.30465	1.7684333	2.66585	0.62695	0.10127
Change in time		19	6.5	16.5	30	24
Change in 8 hours		2.233536842	2.1765333	1.2925333	0.16719	0.03376
Percentage change		#DIV/0!	41.03%	18.27%	1.72%	0.33%



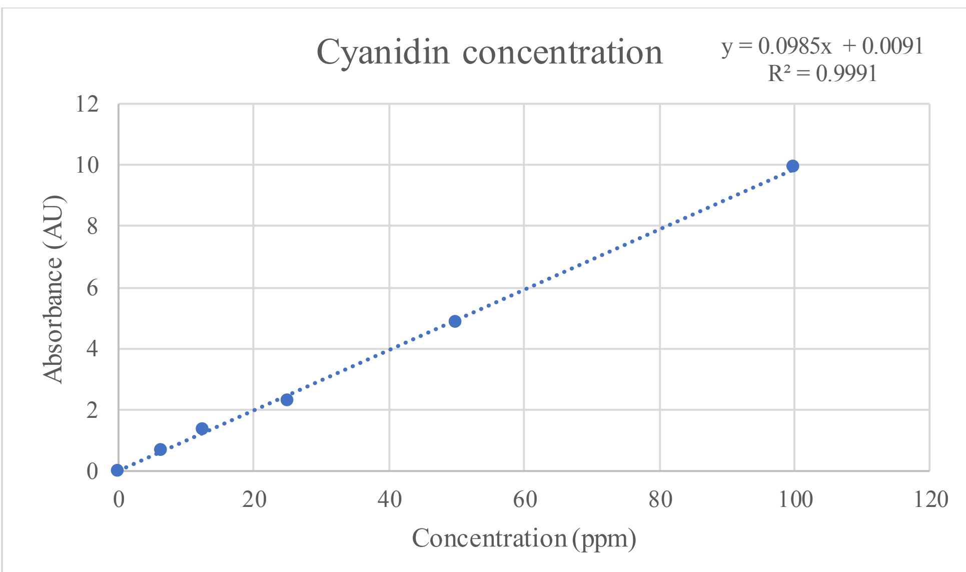
APPENDIX C: Tannin quantification

Standard curve

A standard curve was prepared by using cyanidin as a standard and HCl-Butanol as zero for the absorbance measurement.

Concentration (ppm)	Absorbance (au)	Dilution factor	Corrected Absorbance
100	0.9916	10	9.916
50	0.9761	5	4.8805
25	0.4578	5	2.289
12.5	0.4581	3	1.3743
6.25	0.2288	3	0.6864

$$Concentration = \frac{(Absorbance - 0.0091)}{0.0985} \text{ (in ppm)}$$



The tannin content (TC) was calculated from the data with the following equation:

$$TC = \frac{[(A_S \times C_F) - 0.0091] \times D_V}{10 \times 0.0985 \times M_S \times (1 - M_C)}$$

Where,

TC: tannin content, measured as mg tannin as cyanidin equivalent per 100 g dry sorghum.

A_S: Absorbance from sample (AU)

C_F: Correction factor / Dilution factor

D_V: Dilution volume / Extraction volume (ml)

M_S: Mass of sample (g)

M_C: Moisture content of sample

Tannin content of the samples

Step	Replicate	Abs. (AU)	Dil. Factor	Mass Sample (g)	Dilution Volume (ml)	Moisture Sample	mg Tannin/100 g Sorghum		
							Tannin Content	Average	Std. Dev.
Original sample	A	0.7533	3	0.0637	6	10.82%	241.3487	266.37	24.49
	B	0.6887	3	0.0484	6	10.82%	290.2929		
	C	0.7301	3	0.0557	6	10.82%	267.4779		
After rinse	A	0.8624	2	0.0616	6	22.02%	217.5667	238.23	17.91
	B	0.855	2	0.0533	6	22.02%	249.2777		
	C	0.8342	2	0.0523	6	22.02%	247.8306		
After NaOH treatment	A	0.3894	1	0.0836	6	37.80%	44.54969	48.18	5.70
	B	0.3071	1	0.0645	6	37.80%	45.2461		
	C	0.311	1	0.054	6	37.80%	54.75124		
After final rinse	A	0.1758	1	0.0666	6	30.59%	21.96617	33.77	15.28
	B	0.2434	1	0.0403	6	30.59%	51.02232		
	C	0.2091	1	0.062	6	30.59%	28.30944		

Tannin quantification solids content

Sample ID	Tin	Sample	Final weight	Moisture (%)	Average	SD
Blank	1.2572	0	1.2573			
Grain sorghum	1.3159	2.0345	3.1435	10.17%	10.82%	0.92%
Grain sorghum	1.3131	2.0166	3.0984	11.47%		
After water soak I	1.3205	2.0233	2.8922	22.32%	22.02%	0.46%
After water soak I	1.2906	2.0355	2.8762	22.10%		
After water soak II	1.2933	1.9878	2.8363	22.38%		
After water soak II	1.2963	1.9923	2.8566	21.68%		
After water soak III	1.2992	2.0253	2.894	21.26%		
After water soak III	1.3102	2.105	2.944	22.38%		
After NaOH I	1.2933	2.1022	2.5895	38.34%	37.80%	0.40%
After NaOH I	1.2878	2.0223	2.5367	38.24%		
After NaOH II	1.3023	2.1022	2.6178	37.42%		
After NaOH II	1.2989	1.9858	2.5378	37.61%		
After NaOH III	1.3032	2.0254	2.5695	37.48%		
After NaOH III	1.2908	1.9885	2.5299	37.69%		
After final rinse I	1.2909	2.1761	2.8659	27.62%	30.59%	2.34%
After final rinse I	1.3018	2.1313	2.8253	28.52%		
After final rinse II	1.3057	2.1746	2.8346	29.69%		
After final rinse II	1.2593	2.3378	2.8116	33.60%		
After final rinse III	1.3324	2.1428	2.7929	31.84%		
After final rinse III	1.3181	2.2267	2.8267	32.25%		

APPENDIX D: HPLC Results

100% Untreated sorghum – Without cellulase

Analyte	Ret time	Sample ID											
		13-1	13-2	14-1	14-2	15-1	15-2	16-1	16-2	17-1	17-2	18-1	18-2
Maltose	7.649	0.036	0.032	0.027	0.026	0.032	0.031	0.026	0.024	0.029	0.030	0.035	0.034
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	11.698	0.182	0.168	0.168	0.167	0.173	0.159	0.177	0.176	0.166	0.167	0.183	0.181
Lactic Acid	12.927	0.115	0.106	0.108	0.108	0.120	0.119	0.101	0.101	0.115	0.115	0.122	0.121
Glycerol	13.668	0.975	0.944	0.939	0.936	0.916	0.912	0.924	0.916	0.922	0.931	0.930	0.928
Acetic Acid	15.078	0.004	0.003	0.003	0.002	0.001	0.002	0.003	0.002	0.002	0.002	0.002	0.002
Ethanol	22.129	8.317	8.230	8.111	8.089	7.828	7.792	8.006	7.955	7.930	8.021	8.001	7.981

100% Untreated sorghum – With cellulase

Analyte	Ret time	Sample ID											
		19-1	19-2	20-1	20-2	21-1	21-2	22-1	22-2	23-1	23-2	24-1	24-2
Maltose	7.649	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.712	0.163	0.165	0.158	0.161	0.159	0.159	0.163	0.163	0.162	0.162	0.170	0.169
Succinic Acid	11.698	0.101	0.105	0.102	0.105	0.102	0.106	0.108	0.107	0.103	0.104	0.115	0.112
Lactic Acid	12.927	0.039	0.042	0.040	0.042	0.041	0.040	0.041	0.041	0.039	0.038	0.043	0.042
Glycerol	13.668	0.694	0.711	0.675	0.681	0.677	0.682	0.694	0.698	0.679	0.681	0.719	0.714
Acetic Acid	15.078	0.011	0.006	0.005	0.013	0.005	0.005	0.006	0.005	0.013	0.005	0.013	0.013
Ethanol	22.129	8.893	9.032	8.562	8.657	8.577	8.654	8.750	8.775	8.650	8.626	9.054	9.000

Results expressed as %w/v

100% Corn – Without cellulase

Analyte	Ret time	Sample ID											
		1-1.	1-2.	2-1.	2-2.	3.1	3.2	4.1	4.2	5.1	5.2	6.1	6.2
Maltose	7.621	0.175	0.169	0.159	0.156	0.158	0.156	0.149	0.149	0.154	0.157	0.150	0.150
Glucose	9.196	1.431	1.395	0.148	0.149	0.159	0.163	0.083	0.086	0.494	0.503	0.084	0.084
Succinic Acid	11.616	0.218	0.197	0.204	0.201	0.197	0.195	0.190	0.191	0.190	0.190	0.191	0.193
Lactic Acid	12.831	0.097	0.090	0.079	0.076	0.078	0.074	0.074	0.074	0.075	0.074	0.078	0.080
Glycerol	13.567	1.154	1.106	1.119	1.111	1.088	1.077	1.064	1.069	1.066	1.071	1.096	1.096
Acetic Acid	15.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ethanol	22.088	9.405	9.317	9.631	9.600	9.415	9.295	9.231	9.290	9.247	9.289	9.478	9.483

100% Corn – With cellulase

Analyte	Ret time	Sample ID											
		7.1.	7.2.	8.1	8.2	9.1	9.2	10.1	10.2	11.1	11.2	12-1.	12-2.
Maltose	7.621	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.196	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.866	0.175	0.176	0.174	0.175	0.171	0.169	0.174	0.175	0.172	0.170	0.160	0.160
Succinic Acid	11.616	0.117	0.120	0.119	0.119	0.120	0.120	0.123	0.122	0.121	0.118	0.115	0.114
Lactic Acid	12.831	0.024	0.027	0.000	0.026	0.027	0.027	0.029	0.000	0.028	0.028	0.037	0.038
Glycerol	13.567	0.730	0.732	0.733	0.731	0.721	0.720	0.738	0.735	0.721	0.717	0.702	0.693
Acetic Acid	15.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.021
Ethanol	22.088	11.199	11.160	11.077	11.135	10.981	10.947	11.158	11.039	11.023	11.000	10.923	10.747

Results expressed as %w/v

25% Treated sorghum + 75% Corn – Without cellulase

Analyte	Ret time	Sample ID											
		1-1.	1-2.	2-1.	2-2.	3.1	3.2	4.1	4.2	5.1	5.2	6.1	6.2
Maltose	7.649	0.169	0.165	0.168	0.204	0.177	0.174	0.183	0.176	0.183	0.191	0.151	0.147
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	11.698	0.198	0.181	0.185	0.187	0.178	0.175	0.178	0.175	0.181	0.182	0.195	0.180
Lactic Acid	12.927	0.120	0.109	0.109	0.110	0.104	0.102	0.106	0.106	0.106	0.105	0.134	0.123
Glycerol	13.668	1.112	1.093	1.106	1.108	1.067	1.059	1.082	1.076	1.098	1.100	1.105	1.076
Acetic Acid	15.078	0.008	0.006	0.007	0.008	0.007	0.006	0.006	0.006	0.007	0.006	0.014	0.012
Ethanol	22.129	9.829	10.029	10.058	10.064	9.575	9.541	9.734	9.730	9.935	9.917	9.802	9.738

25% Treated sorghum + 75% Corn – With cellulase

Analyte	Ret time	Sample ID											
		7.1	7.2	8.1	8.2	9.1	9.2	10.1	10.2	11.1	11.2	12-1.	12-2.
Maltose	7.649	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.712	0.188	0.185	0.185	0.181	0.183	0.181	0.184	0.186	0.179	0.180	0.183	0.182
Succinic Acid	11.698	0.145	0.137	0.135	0.129	0.136	0.133	0.137	0.138	0.129	0.131	0.134	0.132
Lactic Acid	12.927	0.050	0.043	0.045	0.043	0.044	0.043	0.049	0.050	0.050	0.050	0.044	0.045
Glycerol	13.668	0.789	0.782	0.777	0.748	0.769	0.760	0.768	0.776	0.732	0.737	0.761	0.757
Acetic Acid	15.078	0.051	0.033	0.035	0.034	0.035	0.033	0.034	0.033	0.031	0.031	0.034	0.034
Ethanol	22.129	11.037	11.184	11.159	10.731	11.041	10.905	11.029	11.129	10.529	10.599	10.935	10.852

Results expressed as %w/v

50% Treated sorghum + 50% Corn – Without cellulase

Analyte	Ret time	Sample ID											
		25-1.	25-2.	26-1.	26-2.	27-1.	27-2.	28-1.	28-2.	29-1.	29-2.	30-1.	30-2.
Maltose	7.649	0.171	0.166	0.150	0.148	0.137	0.148	0.145	0.142	0.152	0.151	0.144	0.137
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	11.698	0.197	0.182	0.178	0.175	0.137	0.187	0.178	0.178	0.179	0.178	0.185	0.183
Lactic Acid	12.927	0.128	0.118	0.110	0.108	0.098	0.118	0.110	0.110	0.114	0.113	0.116	0.115
Glycerol	13.668	1.126	1.098	1.061	1.063	1.064	1.092	1.061	1.062	1.069	1.068	1.079	1.062
Acetic Acid	15.078	0.015	0.013	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.011
Ethanol	22.129	10.014	10.007	9.620	9.668	9.721	9.619	9.588	9.617	9.713	9.693	9.545	9.407

50% Treated sorghum + 50% Corn – With cellulase

Analyte	Ret time	Sample ID											
		7.1	7.2	8.1	8.2	9.1	9.2	10.1	10.2	11.1	11.2	12-1.	12-2.
Maltose	7.649	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.264	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.712	0.202	0.203	0.213	0.211	0.135	0.132	0.210	0.210	0.212	0.219	0.207	0.215
Succinic Acid	11.698	0.129	0.128	0.135	0.134	0.077	0.076	0.134	0.137	0.139	0.141	0.137	0.139
Lactic Acid	12.927	0.043	0.045	0.047	0.047	0.039	0.039	0.045	0.045	0.044	0.046	0.046	0.046
Glycerol	13.668	0.863	0.859	0.878	0.877	0.867	0.855	0.868	0.871	0.880	0.901	0.888	0.884
Acetic Acid	15.078	0.018	0.027	0.030	0.029	0.028	0.027	0.029	0.029	0.031	0.032	0.029	0.029
Ethanol	22.129	11.075	11.082	11.233	11.223	11.205	11.070	11.096	11.125	11.260	11.536	11.404	11.331

Results expressed as %w/v

75% Treated sorghum + 25% Corn – Without cellulase

Analyte	Ret time	Sample ID											
		1-1.	1-2.	2-1.	2-2.	3.1	3.2	4.1	4.2	5.1	5.2	6.1	6.2
Maltose	7.701	0.200	0.202	0.193	0.187	0.180	0.188	0.185	0.198	0.184	0.190	0.182	0.192
Glucose	9.303	0.999	0.978	0.896	0.890	0.741	0.723	0.719	0.743	0.625	0.642	0.374	0.375
Succinic Acid	11.722	0.203	0.189	0.190	0.190	0.204	0.201	0.186	0.195	0.184	0.186	0.190	0.192
Lactic Acid	12.946	0.113	0.103	0.103	0.102	0.111	0.110	0.099	0.102	0.103	0.103	0.102	0.102
Glycerol	13.706	1.104	1.065	1.097	1.088	1.121	1.099	1.062	1.096	1.061	1.077	1.078	1.086
Acetic Acid	15.155	0.017	0.014	0.015	0.015	0.015	0.014	0.015	0.015	0.013	0.013	0.024	0.014
Ethanol	22.186	9.697	9.584	9.902	9.794	9.870	9.636	9.615	9.912	9.355	9.461	9.851	9.824

75% Treated sorghum + 25% Corn – With cellulase

Analyte	Ret time	Sample ID											
		7.1	7.2	8.1	8.2	9.1	9.2	10.1	10.2	11.1	11.2	12-1.	12-2.
Maltose	7.701	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.303	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.988	0.225	0.212	0.206	0.208	0.207	0.205	0.206	0.206	0.212	0.211	0.207	0.212
Succinic Acid	11.722	0.154	0.165	0.151	0.150	0.147	0.146	0.148	0.145	0.152	0.151	0.145	0.150
Lactic Acid	12.946	0.047	0.054	0.045	0.046	0.043	0.042	0.045	0.044	0.044	0.045	0.047	0.048
Glycerol	13.706	0.864	0.901	0.878	0.876	0.856	0.844	0.847	0.834	0.871	0.870	0.843	0.868
Acetic Acid	15.155	0.046	0.050	0.034	0.034	0.031	0.032	0.033	0.032	0.034	0.034	0.030	0.031
Ethanol	22.186	11.130	11.465	11.398	11.368	11.134	11.000	11.033	10.859	11.249	11.309	11.014	11.310

Results expressed as %w/v

100% Treated sorghum – Without cellulase

Analyte	Ret time	Sample ID											
		25-1.	25-2.	26-1.	26-2.	27-1.	27-2.	28-1.	28-2.	29-1.	29-2.	30-1.	30-2.
Maltose	7.701	0.231	0.235	0.234	0.233	0.224	0.232	0.219	0.223	0.210	0.210	0.222	0.224
Glucose	9.303	1.800	1.791	1.900	1.864	1.764	1.798	1.722	1.749	1.685	1.698	1.709	1.740
Xylose	9.988	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Succinic Acid	11.722	0.142	0.145	0.141	0.140	0.135	0.138	0.136	0.137	0.130	0.131	0.141	0.141
Lactic Acid	12.946	0.057	0.058	0.057	0.056	0.054	0.054	0.054	0.055	0.053	0.053	0.055	0.055
Glycerol	13.706	1.100	1.085	1.074	1.058	1.037	1.051	1.043	1.054	0.998	1.004	1.056	1.073
Acetic Acid	15.155	0.020	0.018	0.018	0.018	0.017	0.018	0.017	0.018	0.016	0.016	0.017	0.017
Ethanol	22.186	9.598	9.722	9.606	9.465	9.238	9.370	9.351	9.408	8.901	8.942	9.468	9.601

100% Treated sorghum – With cellulase

Analyte	Ret time	Sample ID											
		31-1.	31-2.	32-1.	32-2.	33-1.	33-2.	34-1.	34-2.	35-1.	35-2.	36-1.	36-2.
Maltose	7.701	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glucose	9.303	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Xylose	9.988	0.220	0.223	0.229	0.240	0.248	0.239	0.240	0.237	0.232	0.233	0.237	0.239
Succinic Acid	11.722	0.142	0.144	0.145	0.146	0.158	0.155	0.152	0.164	0.150	0.149	0.153	0.150
Lactic Acid	12.946	0.041	0.042	0.043	0.044	0.047	0.044	0.044	0.046	0.044	0.043	0.046	0.044
Glycerol	13.706	0.858	0.868	0.876	0.879	0.892	0.910	0.894	0.911	0.890	0.878	0.902	0.888
Acetic Acid	15.155	0.025	0.021	0.027	0.030	0.029	0.029	0.028	0.029	0.027	0.028	0.029	0.028
Ethanol	22.186	10.929	11.008	11.135	11.161	11.297	11.554	11.397	11.617	11.359	11.235	11.429	11.314

Results expressed as %w/v

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