Potential for ethanol production from different sorghum cultivars

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Abstract

This work presents the ethanol production results using three sweet sorghum cultivars. The sugar rich juice was fermented by *Saccharomyces cerevisiae* and *Escherichia coli*. The residual bagasse was further pretreated by dilute phosphoric acid steam explosion. The resulting slurry was submitted to Liquefaction plus Simultaneous Saccharification and co-Fermentation (L+SScF) process using Novozymes Cellic CTec3 enzymes and an engineered ethanologenic *E. coli* strain. Results show a sugar concentration in the juice ranging from 140 to 170 g/L, which were almost completely converted into ethanol by yeast. Concerning the L+SScF, the final ethanol concentration produced increased with enzyme dosage, with little difference among all three sorghum cultivars, reaching up to 27.5 g EtOH/L at enzyme concentrations of 11.5 FPU/gDW. Considering the ethanol produced from juice and from Sweet Sorghum Bagasse (SSB), there is a potential of producing up to 10,600 L of ethanol per hectare, improving on the values reported for corn ethanol.

Keywords

Sweet sorghum, bioethanol, phosphoric acid pretreatment, L+SScF, *E. coli*

1. Introduction

Within the biorefinery concept, bioethanol production continues to be an interesting process because it is still considered to be the most direct and feasible way to partially replace fossil fuels. In addition, bioethanol production presents a number of advantages from the economic, social, and environmental points of view by bolstering the local economy and reducing the amount of carbon dioxide released into the atmosphere.

The possibilities of using local feedstocks or dedicated energy crops make the ethanol production option more attractive, as it can also contribute to technical and economic development of rural areas. In this context, sweet sorghum has attracted attention because it can compare favorably with other energy crops such as corn or sugar cane when cultivated in marginal areas, while yielding a similar amount of fermentable sugars. Some of the advantages of using sweet sorghum include that it can be cultivated twice a year in diverse climates, has a low requirement for fertilizer, high efficiency in water usage, and the potential to be drought resistant (Erickson et al., 2011; Whitfield, Chinn and Veal, 2012; Adams et al., 2015).

A number of studies have been devoted to assessing sweet sorghum performance in agronomic terms (Linton et al., 2011; Davila-Gomez et al., 2011; Fernandes et al., 2014). These studies were mostly focused on the ethanol production derived from the soluble sugars contained in the juice (Yu et al., 2012). The sweet sorghum juice is obtained from squeezing the sorghum stalks, with the main sugar being sucrose. On the other hand, the remaining solids after extracting the juice (sweet sorghum bagasse, SSB) constitute a lignocellulosic residue whose use as raw material for ethanol production is advantageous due to the lack of competition with food applications and its relatively high sugar content (as cellulose and hemicellulose).

In order for lignocellulosic biomass to be converted into ethanol, the polymeric sugars need to be solubilized through pretreatment and enzymatic hydrolysis, followed by microbial fermentation. We have developed a simplified process termed liquefaction plus simultaneous saccharification and co-fermentation (L+SScF), coupled with dilute phosphoric acid pretreatment, that has been successful in the production of

lignocellulosic ethanol from sugarcane bagasse (Nieves et al., 2011b), SSB (Wang et al., 2015), and eucalyptus chips (Castro et al., 2014) at high ethanol yields.

The main objective of this work was to assess the possibilities of using three new sweet sorghum cultivars developed at the University of Florida as raw material for bioethanol production, considering both the juice and bagasse, from agricultural production to fermentation. Special attention was placed on lignocellulosic ethanol and the effect of enzyme dosage during liquefaction on the overall ethanol yield. In addition, an ethanologenic *Escherichia coli* strain, capable of converting both hexoses and pentoses, was used as the biocatalyst during fermentation.

2. Materials and methods

2.1 Plant material

2.1.1 Sorghum juice

Three University of Florida sweet sorghum [*Sorghum bicolor* (L.) Moench] cultivars – F_6(Honey × Bk7)-45-3-1-1-1, F_6(Mer81-4 × Bk7)-20-2-1-1-1 and F_6(Mer81-4 × Bk7)-15-2-1-1-1, referred to from here on as UF45, UF20 and UF15, respectively, were cultivated at the Plant Science Research and Education Unit near Citra, FL (29.410629 N, 82.170081 W) during the spring and summer of 2012. These cultivars were selected using the pedigree method with the primary selection criteria being the yield of soluble sugars in the stem juice, biomass yield, and resistance against the fungal disease anthracnose (Felderhoff et al., 2016). The fields were fertilized with 250 kg ha^{-1} of a liquid fertilizer (10-34-0) at planting. An additional 125 kg ha^{-1} of N and K_2O were applied after planting. Three weeks after emergence, seedlings were thinned to 10 plants per row meter giving a plant population of approximately 131,600 plants ha^{-1}. Insecticides were used as

needed to limit the damage from fall armyworm, aphids, or other pests. When the plants had reached the hard-dough stage of maturity (seeds no longer able to be squeezed between fingers), for each genotype a row (7.6 m) of plants was cut, leaves and panicles were removed, and the stems were pressed in a roller mill to extract the juice, which was collected in a bucket. A fresh sample of the juice was obtained for sugar analysis and the remainder was stored at -20 ºC in sealed 20-L buckets until needed.

2.1.2 Sorghum bagasse

These same three cultivars were planted in a commercial field managed by Delta BioRenewables, LLC, near Memphis, TN on 20 June 2014 and harvested on 24 September 2014. The sweet sorghum was harvested with a forage chopper, pressed with a commercial two-roller press (Laurel Machine and Foundry, Laurel, MS), imbibed with water to extract additional soluble sugars, then pressed a second time, imbibed again, and pressed a third time. The bagasse was then dried with hot air in a peanut wagon and shipped by truck to the Stan Mayfield Pilot Biorefinery in Perry, FL.

2.2 Sweet sorghum juice fermentation

2.2.1 Yeast fermentations

Prior to use for fermentation, the juice was thawed, boiled for 5 min, and then cooled to room temperature. Inoculum for fermentations using yeast came from Prestige Turbo Pure 48 Turbo Yeast (Gert Strand AB, Svedala, Sweden). Yeast was proofed in 100 mL water in a 500 mL flask, 1 g yeast was added and the culture was grown at 35 ºC, 100 rpm for 20 minutes in a New Brunswick shaker incubator. Sorghum juice was supplemented with 2.5 g/L urea. Using 500 mL fleakers, 300 mL of sorghum juice was

inoculated with 2% (v/v) proofed yeast. Fleakers were grown at 30 ºC, 150rpm, with no pH control.

2.2.2 *E. coli* fermentations

The *E. coli* strains SL200A (XW055pLOI2751-T41), SL300 (LY180-T18) and SL400 (XW068-T26) were used for testing the fermentability of the sorghum juice. Cultures were initially grown in standing screw-capped tubes with 5% sucrose and AM1 mineral salts medium at 37 ºC. Strains SL200A and SL400 had 100 mM MOPS (pH 7.0) added and SL200A had 100 mM $KHCO_3$. Seed fleakers were inoculated from tubes containing the same media without MOPS. The sorghum juice was diluted to 100 g sugar/L with AM1 salts plus trace elements and water. The pH was controlled with 4:1 3M K_2CO_3:6M KOH (SL200A, pH 7.0), 2M KOH (SL300, pH 6.5), and 6M KOH (SL400, pH 7.0). Cultures were grown at 37 ºC, 150 rpm.

To get optimal sucrose utilization for strains LY180 (*E. coli* W ethanol strain, (Geddes et al., 2011) and XW068 (Wang et al., 2011) cultures were transferred in fleakers containing 10% sucrose with AM1 mineral salts medium. After 18 transfers with LY180 at 37 ºC, 150 rpm and pH 6.5, SL300 was isolated. After 26 transfers with XW068 at 37 ºC, 150 rpm and pH 7.0, SL400 was isolated. Strain XW055 (*E. coli* C succinate strain, (Wang et al., 2013)) has no native pathway for sucrose utilization. The sucrose operon, cscA-cscK-cscB (invertase, fructokinase, permease, respectively) was cloned from *E. coli* W into vector pTrc99a (Amann, Ochs and Abel, 1988), using PCR (Pfx50, Invitrogen, Carlsbad, CA) and the NdeI and XbaI sites, making pLOI5720. Plasmid pLOI5720 was digested with AhdI (Klenow treated, New England BioLabs, Ipswich, MA) and XmnI and self ligated to make pLOI5721. This deletes the bla gene,

leaving no antibiotic resistance marker on the plasmid. Plasmid pLOI5721 was then transformed into strain XW055 and transferred in fleakers containing 10% sucrose with AM1 mineral salts medium plus 100 mM $KHCO_3$. After 41 transfers with XW055(pLOI5721) at 37 ºC, 150 rpm and pH 7.0, SL200A was isolated (Table 3).

2.3 Phosphoric acid steam explosion pretreatment

Phosphoric acid pretreated bagasse was prepared at the University of Florida Stan Mayfield Biorefinery (0.5% (w/w) phosphoric acid on a dry biomass basis, 5 min, 190°C) as previously described (Nieves et al., 2011b) using a steam pretreatment device (Linde, Galbe and Zacchi, 2007; Palmqvist et al., 1996). After steam pretreatment, the discharged fiber contained ~70% moisture (~30% dry weight including fiber and solubles). Multiple pretreatment runs (15-20 runs at 0.5 kg each bagasse dry weight) were blended to make each batch, and stored at -20 ºC. This material was either used directly for liquefaction plus simultaneous saccharification and co-fermentation (L+SScF) (Geddes et al., 2011), or fractionated into liquid hemicellulose hydrolysate (used for seed growth) and fiber (discarded) with a model CP-4 screw press (Vincent Corporation, Tampa, FL).
For experimental convenience, fine particulates were removed from the pressed hydrolysate using a glass fiber filter (Whatman GF/D, 15 mm diameter, 27 µm pore size). The clarified hydrolysate was stored at 4 ºC until needed.

2.4 Liquefaction plus Simultaneous Saccharification and co-Fermentation (L+SScF)

Water was added to phosphoric acid pretreated SSB (10% dry wt solubles and fiber, final concentration after inoculation), adjusted to pH 5 with 5 N ammonium hydroxide, mixed with cellulase and incubated for 6 h at 50 ℃ to allow liquefaction.

Novozyme Cellic CTec3® cellulase was used at three different concentrations (2.88, 5.75, and 11.5 FPU/gDW, corresponding to 1.25, 2.50 and 5.00 % v/w respectively) based on the SSB dry weight after inoculation. The liquefaction step was conducted in 1 gal freezer bags immersed in a water bath with hourly manual mixing. Contents were transferred to 2-L BioFlo 110 fermentors, cooled to 37 ^{O}C, and adjusted to pH 6.3 with 5 N ammonium hydroxide. Trace metals and magnesium sulfate salts were added according to the recipe for AM1 media (Martinez et al., 2007) and sodium metabisulfite was added to provide a final concentration of 1.0 mM (Nieves et al., 2011a). The simultaneous saccharification and co-fermentation was initiated by adding 10% (v/v) inoculum of a hydrolysate-resistant strain of *E. coli* SL100 from a 2-L seed fermentor and monitored for up to 96 h at 37 ^{O}C. During the seed growth, SSB clarified hydrolysate was used and prepared as stated before (Geddes et al., 2013). Small amounts of air (0.01 vvm, 20 mL/min (Nieves et al., 2011b)) were added throughout the fermentation.

2.5 Analytical methods

The composition of the raw material was determined according to National Renewable Energy Laboratory (NREL) analytical methods for biomass (Sluiter et al., 2008). Monomer sugars (glucose, xylose, arabinose, mannose and galactose) and inhibitor composition (acetic acid, formic acid, furfural and HMF) of the liquid fraction were determined by HPLC using an Agilent Technologies 1200 series HPLC system as described in Geddes et al. (Geddes et al., 2011). Ethanol was measured using an Agilent Technologies 6890N Network gas chromatography system (Geddes et al., 2011). Dry matter was determined using a Kern model MLB 50-3 moisture analyzer (Balingen,

189 Germany). All analytical determinations were performed in triplicate and the average
190 results are shown. Relative standard deviations were below 3%.
191
192 **3. Results and discussion**
193 *3.1 Raw material composition*
194 Table 1 depicts the composition of the three SSB cultivars used in this study.
195 Sugars polymers account for approximately 2/3 of the dry weight. Glucan represents
196 more than 40% of the dry weight, while xylan is the most important hemicellulosic
197 polymer in SSB, followed by arabinan. This composition is in accordance with other
198 previously reported values (Shen et al., 2011; Wang et al., 2012; Li et al., 2010) and
199 confirms SSB as a lignocellulosic material of interest for ethanol production.
200
201 *3.2 Biochemical production from juice*
202 The composition of the soluble sugars in the sorghum juice is shown in Figure 1A.
203 With all three sorghum cultivars, sucrose was present in the highest concentration, with
204 UF20 producing the most amongst them. On the other hand, UF45 had the lowest
205 concentration of sucrose and the lowest concentration of total sugars released. The juice
206 of UF45 contains proportionally more monosaccharides and less sucrose compared to
207 UF15 and UF20, which matches the differences in the profiles of the sweet sorghum
208 parents: 'Honey' (UF45) is an amber type, historically cultivated for the production of
209 syrup, whereas 'Mer81-4' (UF15) is a more modern sweet sorghum cultivated for the
210 production of sugar for industrial uses.
211 The sorghum juice obtained from all three cultivars was fermented using turbo
212 yeast (for ethanol production) and three separate strains of *E.coli* that had been

213 engineered for the production of ethanol, succinic acid, and lactic acid. The yield for
214 ethanol production using turbo yeast ranged from 87-93%, which compares well with the
215 results reported using high sugar concentrations and S*accharomyces cerevisiae* NP 01
216 under optimal aeration conditions, where 127.8 g ethanol/L were produced from 280 g
217 total sugars/L, equivalent to 89% of theoretical ethanol production (Deesuth, Laopaiboon
218 and Laopaiboon, 2016). Other authors also reported average fermentation efficiencies of
219 85% for the ethanol production form five different sorghum cultivars (Davila-Gomez et
220 al., 2011).

221 On the other hand, the production of ethanol from *E. coli* SL300 varied between
222 75-102% (Figure 1B). The succinate fermentations resulted in the lowest yields. It is
223 interesting to note that all *E. coli* fermentations had lower yields when using the juice
224 obtained from UF20. This lower yield might be related to the higher levels of sucrose
225 present in the UF20 juice.

226

227 *3.3 Sweet sorghum pretreatment results*

228 The characterization of the phosphoric acid steam explosion pretreated SSB is
229 shown in Table 1. As expected, the pretreatment caused a sharp decrease of the
230 hemicellulose content (particularly xylan, as the major hemicellulosic polymer) when
231 compared to the untreated raw material (Table 1). As a consequence, an increase of the
232 concentration of glucan and lignin is detected. The solubilization of xylan has been
233 reported as one of the reasons of improving cellulose accessibility to enzymes (Himmel et
234 al., 2007) which in turn results in higher glucose concentrations and, finally, greater
235 ethanol conversions. Similar results were obtained for steam explosion of SO_2-
236 impregnated SSB at 190 ºC for 5 min, where xylan composition dropped from 19.4 to

9.8% in the pretreated solids (Shen et al., 2012). In addition, arabinan content was also reduced as a consequence of the pretreatment. The composition of our pretreated solids, with respective average values of 53.2, 8.2, and 27.7% for cellulose, hemicelluloses, and lignin, is also very close to the one reported by (Pengilly et al., 2015) in a study of SSB pretreated with steam at 200 ºC for 5 min (52.4, 9.4, 25.0%, for cellulose, hemicelluloses, and lignin respectively).

The composition of the liquid fractions issued from pretreatment is shown in Fig. 2A. The recovery of sugars in the liquid fractions, defined as the fraction of sugar initially present in the raw material that is found in the liquid after pretreatment, reveals that 40, 35 and 55% of the initial xylose (21, 18 and 25% of all sugars) is recovered in the liquid fraction when using cultivars UF15, UF20, and UF45, respectively. With respect to glucose, an average of 8% enters the liquid phase after pretreatment, indicating that some hydrolysis of the cellulose fraction took place as a consequence of the pretreatment.
In addition to the sugars released, other compounds are also present in the liquid fraction as a result of sugar degradation and hemicellulose hydrolysis during pretreatment. These compounds can have a negative impact in the process as they act as inhibitors of the fermentation biocatalyst (Zaldivar, Martinez and Ingram, 1999). Acetic acid and furan derivatives, with furfural and hydroxymethylfurfural (HMF) as prominent examples, have been described as the main inhibitory compounds released during the hydrothermal pretreatment of lignocellulose materials (Jönsson and Martín, 2016). Acetic acid appears as a consequence of the breakdown of the acetyl bonds that form hemicellulose, while furfural and HMF form from the dehydration at high temperature and low pH of pentose and hexose sugars respectively. Levulinic and formic acids can also be obtained from further degradation of the furan compounds (Jönsson and Martín, 2016). In addition to

the abovementioned compounds, lactic acid is also reported as appearing in the liquids from hydrothermal pretreatment of sweet sorghum stems (Sun et al., 2015).

Figure 2B shows the composition of the liquids in terms of inhibitors. Acetic acid had the highest concentration on hydrolysate obtained from pretreated UF45 (3.6 g/L or 12.1 g/kg). This was to be expected, as this was also the variety with the highest hemicellulose hydrolysis (as can be observed by the higher xylose concentration, Figure 2A). These results are consistent with the ones reported by other researchers. For example, acetic acid concentrations of 5.3 g/L were found in the liquid fraction obtained after 200 ºC steam explosion pretreatment for 5 min, as well as minor concentrations of furfural and HMF (Zaldivar, Martinez and Ingram, 1999). After acetic acid, furfural was also detected in the liquids from pretreatment at concentrations ranging from 1.28 to 1.47 g/L (3.1 to 4.0 g/kg), followed by lower amounts of HMF and formic acid. Following a similar pattern to the sugar release, the SSB obtained from cultivar UF20 was the one producing the lowest concentration of inhibitors in the liquids, with 15.5 total inhibitors (sum of acetic acid, furfural, HMF, and formic acid) per kg SSB (5.8 g/L).

3.4 Liquefaction plus simultaneous saccharification and co-fermentation (L+SScF)

Following pretreatment, the whole slurry was further submitted to a liquefaction step using Cellic-Ctec3 enzymes (230 FPU/mL) for 6 h and then adding *E. coli* for simultaneous saccharification and co-fermentation of sugars present in the slurry. Figure 3 depicts the final concentration of the main sugars attained at the end of the 6-h liquefaction step, at the different enzyme concentration tested (1.25, 2.50 and 5.00%, corresponding to 2.88, 5.75, and 11.5 FPU/g DW biomass respectively). This figure shows the clear effect of increasing enzyme concentrations on sugar release, no matter

the type of sugar or the SSB variety. Although this effect is more evident on glucose release, the enzyme complex also exhibits xylanase activity, as shown by the increasing xylose concentration. For the different SSB cultivars, UF20 was the one with the highest concentration of total sugars released, although the differences among all three cultivars were relatively small, especially at the higher enzyme dosage.

After a 6-h liquefaction, the slurry was inoculated with *E. coli* SL100 and the simultaneous saccharification and co-fermentation of sugars was monitored. As an example, the time evolution of the main sugars as well as that of the fermentation products is presented in Figure 4A for cultivar UF15 using an enzyme concentration of 11.5 FPU/gDW. Similar profiles were obtained for all three sorghum cultivars tested.

As can be seen, all sugars (except galactose) were completely consumed during the process. Glucose was depleted in all cases at 48 h or less (for the lower enzyme dosages), while the consumption of xylose took up to 72 h in the cases of higher initial sugar content. It is also worth noting that furfural, even if it was found in lower concentration than minor sugars, was also consumed at the first stage of the SScF process, and its depletion seems to initiate the consumption of glucose and xylose. This behavior has also been described for other microorganisms like *Neurospora crassa*, fermenting SSB hydrolysate (Dogaris et al., 2012) or *S. cerevisiae* (Almeida et al., 2009) and is attributed to the conversion of furfural to other less inhibiting compounds such as furoic acid or furfuryl alcohol. However, as the enzyme concentration was reduced, the final concentration of ethanol was also reduced, reaching highest values of 16.4, 22.4 and 27.5 g/L for 2.88, 5.75, and 11.5 FPU/gDW of enzyme concentrations respectively (Figure 4B). This is to be expected, as there would be less sugars available for the fermentation.

To take into account the effectiveness of the pretreatment and L+SScF process, the overall ethanol yield for the nine cases under study was calculated (Figure 5). The results show that there is a marked increase in terms of overall ethanol yield when doubling the enzyme loading from the lowest to the intermediate level assayed (37.1, 41.7 and 38.5% yield increase for UF15, UF20 and UF45 respectively). However, when doubling the enzyme concentration once again to 11.5 FPU/g DW, the increase is not as high. In this case, the UF15 variety ethanol yield increased by 22.7% while the other two cultivars improved this parameter by only 7.1% and 11.8% (for UF20 and UF45 respectively).

3.5 Potential for ethanol production

Sweet sorghum has the potential to be an effective feedstock for ethanol production. Grains, with high starch content, are a sugar source for ethanol. The crushed stalks generate a sucrose rich juice that can also be converted to ethanol by hexose fermenting microorganisms. And finally, the bagasse obtained after juice extraction is a lignocellulosic material with high sugar content in the form of cellulose and hemicellulose, which can be deconstructed to hexoses and pentoses, and be further converted into ethanol by fermentation. Grains, juice, and bagasse account for approximately 5, 55, and 35% of the mass balance of sweet sorghum produced per hectare (Barcelos et al., 2016) (the remaining being leaves and straw, which are much more difficult to include in the ethanol production process, and usually left in the fields).

Table 2 presents a comparison of results obtained with SSB under a wide range of operational conditions, covering different types of pretreatment methods, enzymes and fermentative microorganisms. An interesting note is that the publications with the highest

reported values for ethanol yield (of those listed in Table 2), all included a washing step after pretreatment in order to remove inhibitors and facilitate the bioconversion of sugars to ethanol (Darkwah, Wang and Shahbazi, 2016; Dogaris et al., 2012; Li et al., 2013; Wang, Luo and Shahbazi, 2013). Although the breadth of the experimental conditions makes it difficult to establish direct comparisons, our results are in line with those of other researchers.

Taking a closer look at the potential for ethanol production from the sorghum cultivars analyzed in the present work, the amount of ethanol possible from the juice and the fiber compares favorably with the amounts of ethanol currently produced from corn grain. Based on the yields obtained from field tests, it would be possible to generate over 6,300 L EtOH/ha for UF15, over 5,500 L EtOH/ha for UF20, and over 5000 L EtOH/ha for UF45, considering only the sugars produced from the juice. In 2014, corn ethanol averaged some 4200 L/ha (Goldemberg and Guardabassi, 2010), so the sorghum ethanol yields are higher for all three cultivars assayed. If the residual lignocellulosic material after juice extraction is also taken into account, our results show that additional amounts of ethanol of 4,163 L EtOH/ha for UF15, 3,154 L EtOH/ha for UF20, and 3,299 L EtOH/ha for UF45 can be produced. The comparison can be established also with the lignocellulosic residues of corn, e.g., corn stover. The production of corn stover has been estimated to be in a 1:1 mass ratio of corn grain (Tumbalam et al., 2016). Based on a recent report which assumed that only 50% of the produced corn stover is harvested (because of the well-known benefits of retaining a part of the corn stover as a soil amendment), a production of 1,473 L of EtOH/ha can be obtained. Even if we were to consider 100% of the corn stover to be used for lignocellulosic ethanol production, it is still a smaller amount than the one derived from SSB of any of the cultivars assayed in

this study (2,946 for corn stover vs 3,154 for SSB). As shown in Figure 6, the total yield of ethanol from both origins, is higher by 47%, 22% and 17% when comparing UF15, UF25 and UF45 with corn. Additionally, SSB is already found as a by-product in the location where the juice is extracted, so in comparison with corn stover, SSB represents an economic advantage in terms of collection and transportation costs.

Although the information on bioethanol production based on the cultivated area of sweet sorghum is seldom available in the scientific literature, some authors still offer this information, sometimes based on laboratory experimental results and on theoretical conversion yields. The wide range of conditions for the different steps of the process makes difficult a direct comparison. Nevertheless, our results are similar to the ones recently reported using sulfuric acid pretreatment and *S. cerevisiae* to convert sugars obtained from the juice and a flocculant strain of *Scheffersomyces stipitis*, which was the fermentative microorganism for sugars from bagasse. The potential for ethanol production was estimated to be up to 11200 L/ha (Barcelos et al., 2016), without considering the additional ethanol that could be obtained from sweet sorghum grains. The production of a number of chemicals including bioethanol, butanol, and degradable wood plastic composites under the biorefinery concept has been proposed in an attempt to overcome the seasonal availability of sweet sorghum (Yu et al., 2012).

4. Conclusions

Sweet sorghum is an excellent raw material for the production of bioethanol, presenting several advantages, such as its high productivity and its relative resistance to harsh conditions. Fermentable sugars are obtained from the juice (mainly in form of sucrose) and from the bagasse produced after juice extraction (mainly in form of glucose and

xylose). While sugars from juice can be fermented to ethanol by a simple process, the bagasse needs to be submitted through a complex process involving a pretreatment step and an enzymatic hydrolysis to produce a mixture of pentose and hexose sugars which can then be fermented. In the present work, three varieties of sweet sorghum were assayed for ethanol production. The fermentation of sugars from the sweet juice was successful using either common industrial biocatalysts (*S. cerevisiae*), as well as engineered microorganisms (*E. coli*). In addition, the SSB was further processed using a phosphoric acid pretreatment, followed by L+SScF with an ethanologenic E. coli strain as biocatalyst. Results showed that all the three assayed varieties produced between 8300 and 10500 L ethanol/ha from the combined conversion of sugars from the juice and SSB.

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533

534 **Table 1.** Composition (average values and standard deviations of three determinations)
535 of the raw sweet sorghum bagasse and its washed pretreated solids used in this study.

Component /Cultivar	UF15		UF20		UF45	
	Raw	Pretreated	Raw	Pretreated	Raw	Pretreated
Glucan	44.4 ±0.86	52.7±2.0	42.4±0.25	51.7±2.3	44.5±2.01	55.2±0.45
Xylan	19.5±0.84	8.5±0.30	18.7±0.26	8.1±0.28	19.0±0.82	8.0±0.06
Arabinan	3.2±0.32	1.0±0.64	2.5±0.13	1.8±0.64	2.7±0.020	0.84±0.07
Lignin	19.6±0.27	27.7±0.88	22.0±0.63	27.5±0.70	22.2±0.18	27.8±0.37
Acetate	2.6±0.72	1.36±0.13	2.7±0.13	1.4±0.30	2.9±0.37	1.5±0.73

536

537

538 **Table 2.** Comparison of results obtained using sweet sorghum bagasse under different

539 process schemes

Pretreatment conditions	Enzymes/ Fermenting microorganisms	Main results	Reference
SO_2-steam explosion	• Spezyme-CP and β-glucosidase • *S. cerevisiae*, Tembec T1	153 g EtOH/kg SSB without xylose fermentation	(Shen et al., 2011)
Hydrothermal pretreatment by microwave digestion	• Cellic CTec2 • Baker yeast	230 g EtOH/kg SSB	(Matsakas and Christakopoulos, 2013)
2% v/v H_2SO_4 acid, 75ºC and then 121ºC	• Cellic Ctec, Cellic Htec, Promalt 295, Promalt 4TR • *P. tannophilus* and *S. cerevisiae* DCLM	23 g/L ethanol (72% of theoretical yield)	(Nasidi et al., 2015)
180ºC, 0.5% sulfuric acid	• Cellulase, β-glucosidase and hemicellulase • *S. cerevisiae* ATCC 24858	The ethanol yield, concentration and production rate were 89.4%, 38 g/L and 1.28 g/L/h, respectively	(Wang, Luo and Shahbazi, 2013)
Ammonium fibre explosion (AFEX) at 140 C for 30 min	• Cellulase (Spezyme CP) and xylanase (Multifect xylanase) • *S. cerevisiae* 424A (LNH-ST)	42.3 g/L EtOH 159 g EtOH/kg SSB	(Li et al., 2010)
2% SO_2 Steam explosion at 180-200 °C for 5-10 min	• Celluclast 1.5 L and Novozym 188 • Baker yeast	85-90% conversion in pretreatment 173 g EtOH/kg SSB	(Sipos et al., 2009)
Dilute acid microwave assisted pretreatment	• Celluclast 1.5 L, and β-glucosidase • *Neurospora crassa*	345 g EtOH/kg SSB	(Dogaris et al., 2012)
Advanced Solid State Fermentation+ Distillation and NAOH treatment	• Cellic Ctec-3 • *S cerevisiae* TSH1/ *Zymomonas mobilis* TSH-01	92 g EtOH/kg fresh sweet sorghum stalks equivalent to 328 g EtOH/kg SS dry basis (juice and SSB)	(Li et al., 2013)

5% acetic acid+0.5% sulfuric acid at 180ºC for 5 min	• Cellulases, beta-glucosidases and hemicellulases • *S. cerevisiae*, ATCC 24858	Fed batch SSF for 96 h at 20% solids concentration produced 53.1 g/L ethanol (88.7% yield) compared to 25.7 g/L and 86.7% yield at 10% solids loading	(Darkwah, Wang and Shahbazi, 2016)
Steam explosion impregnated with H_3PO_4	• Cellic Ctec-3 • *Escherichia coli* SL100	Effective fermentation of hexoses and pentoses. 275 g EtOH/kg dry SSB	This study

540

541

542 **Table 3.** Plasmids and primers

Plasmids			Reference
pTrc99a	*bla oriR rrnB lacIq*		(Amann, Ochs and Abel, 1988)
pLOI5720	*cscA-cscK-cscB* in pTrc99a, deletes *lacIq*		This study
pLOI5721	pLOI5720 digested with AhdI and XmnI, deletes *bla*		This study
Primers			
EC-cscKBA-f	AATCTAGAGACCGTGATACACGGGACAG	XbaI site added	(Chan, Kanchanatawee and Jantama, 2012)
suc-cscA 3	GAGCATATGACTACACCGATCTCGCAAGT	NdeI site added	This study

543

Figure captions

Figure 1. A) Sugar concentrations in the juice of the different sorghum cultivars. B) Yield on a weight/weight basis obtained from the juice.

Figure 2. Composition of liquids (g/kg SSB, dry matter) released from phosphoric acid-soaked, steam exploded sweet sorghum bagasse. A) Sugars. B) Inhibitors.

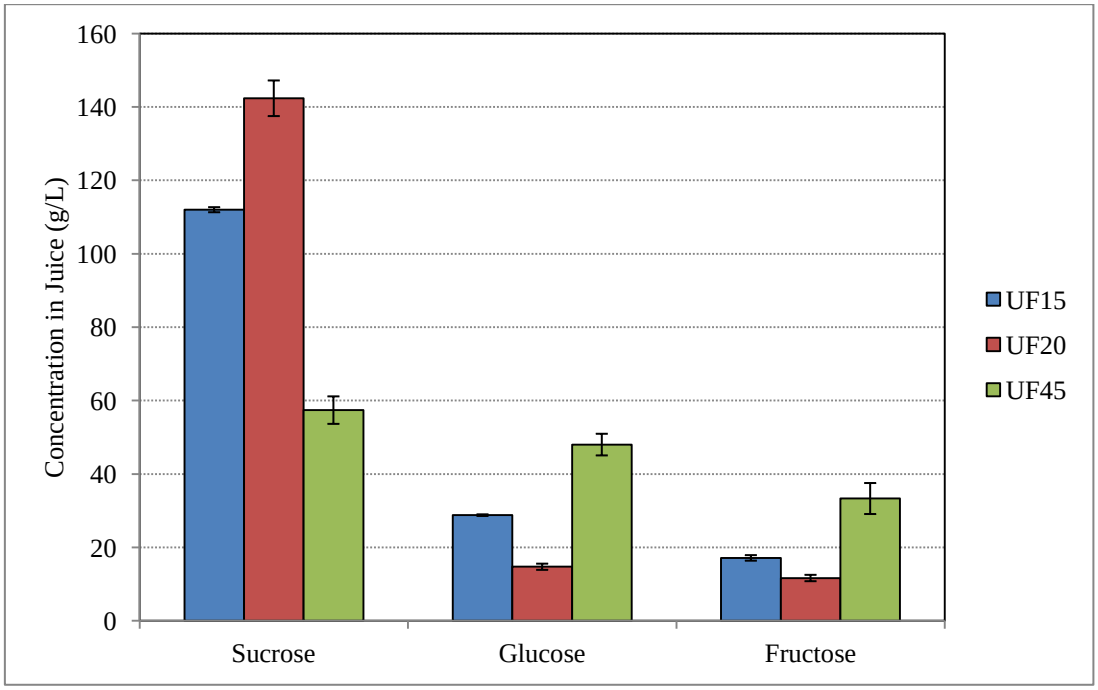
Figure 3. Initial sugar composition of SScF after a 6 h-liquefaction as a function of sorghum cultivar and enzyme concentration. Values in the x-axis refer to the concentration of enzyme used in FPU/g DW.

Figure 4. A) Time evolution of sugars, furfural and ethanol during simultaneous saccharification and co-fermentation of pretreated UF15 SSB slurry using *E. coli* SL100 and 11.5 FPU/g DW of Cellic CTec3 enzymes. B) Ethanol concentration for all three sorghum cultivars using varying concentrations of enzyme.

Figure 5. Overall ethanol yield using varying concentrations of enzyme. Error bars represent the standard deviations of at least 4 replicate experiments.

Figure 6. Potential ethanol production from the three sorghum cultivars assessed in this study and the average yield for corn ethanol from the year 2014. Juice and fiber correspond to sweet sorghum and grain and stover to corn.

561 A)



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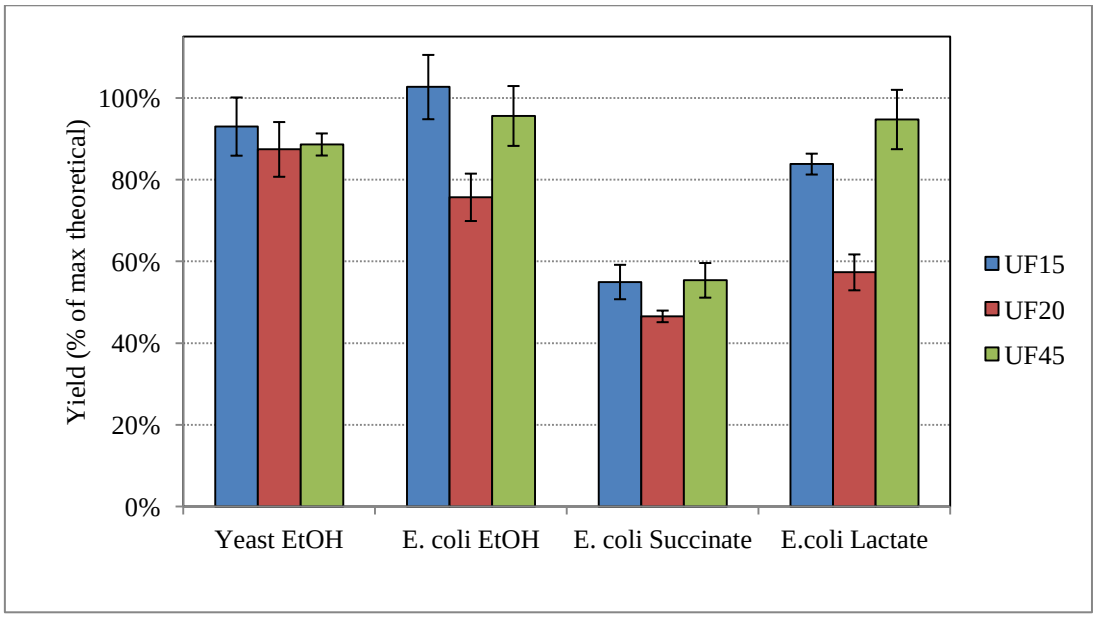
563 B)

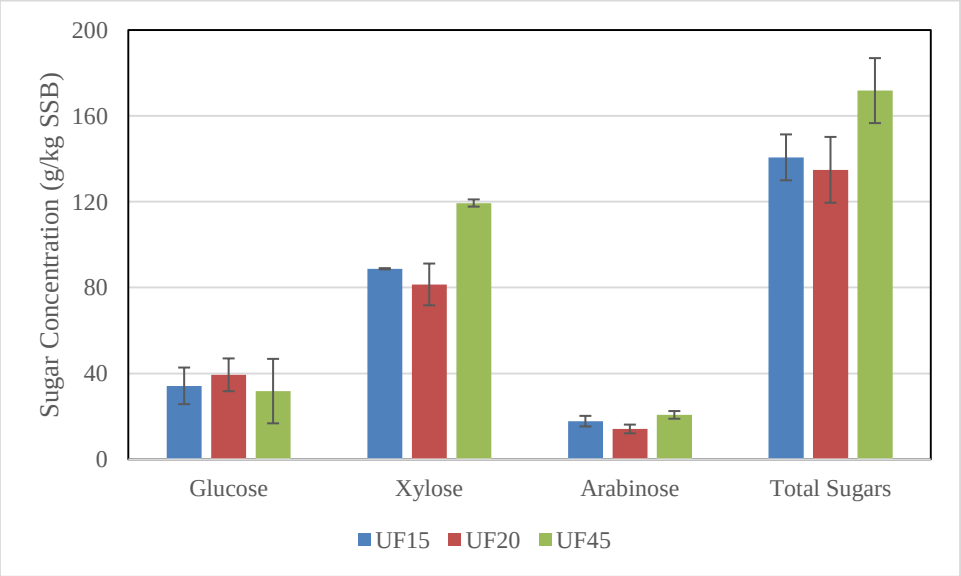


564

565 **Figure 1.**

566

A)


Sugar Concentration (g/kg SSB)
200
160
120
80
40
0
Glucose
Xylose
Arabinose
Total Sugars
UF15
UF20
UF45

B)

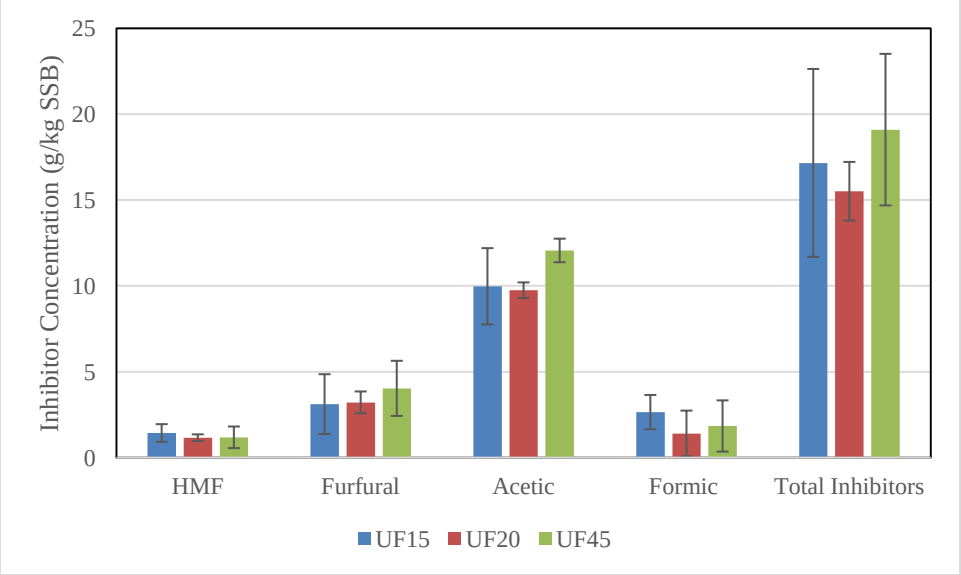

Inhibitor Concentration (g/kg SSB)
25
20
15
10
5
0
HMF
Furfural
Acetic
Formic
Total Inhibitors
UF15
UF20
UF45

Figure 2.

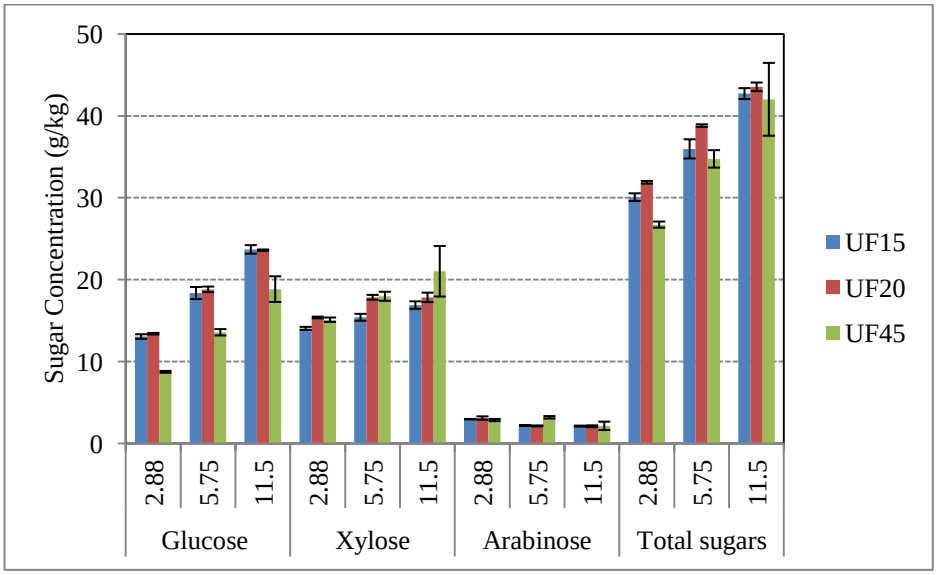
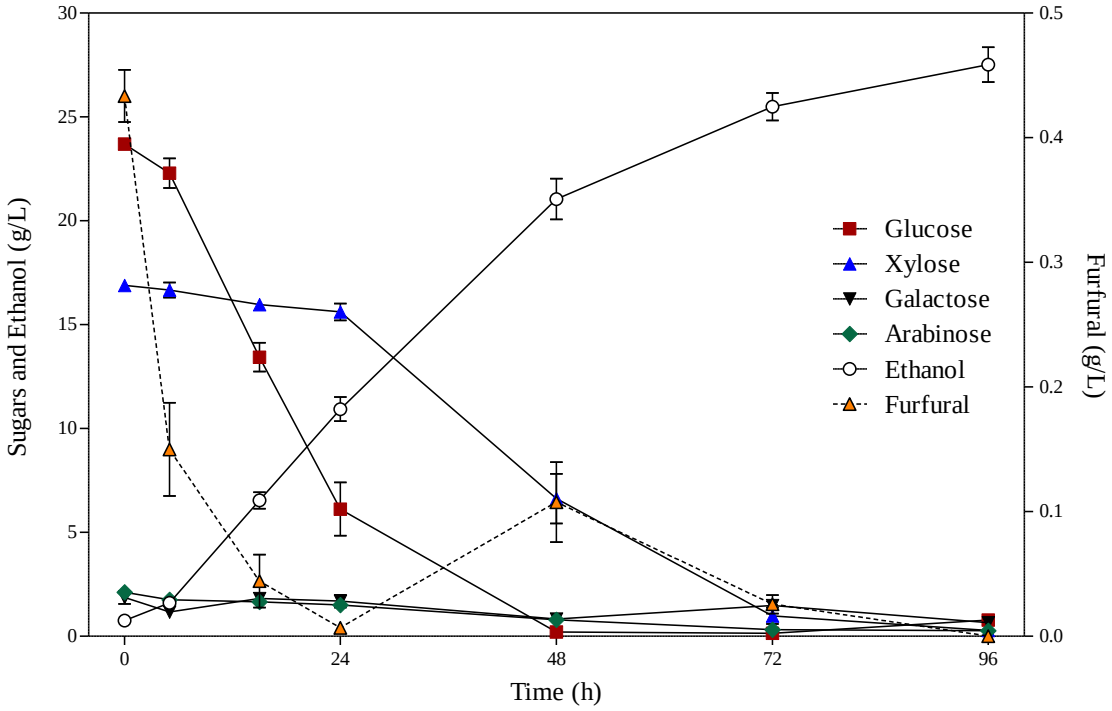

Sugar Concentration (g/kg)
50
40
30
20
10
0
2.88
5.75
11.5
Glucose
Xylose
Arabinose
Total sugars
UF15
UF20
UF45

Figure 3.

577 A)



578

579 B)

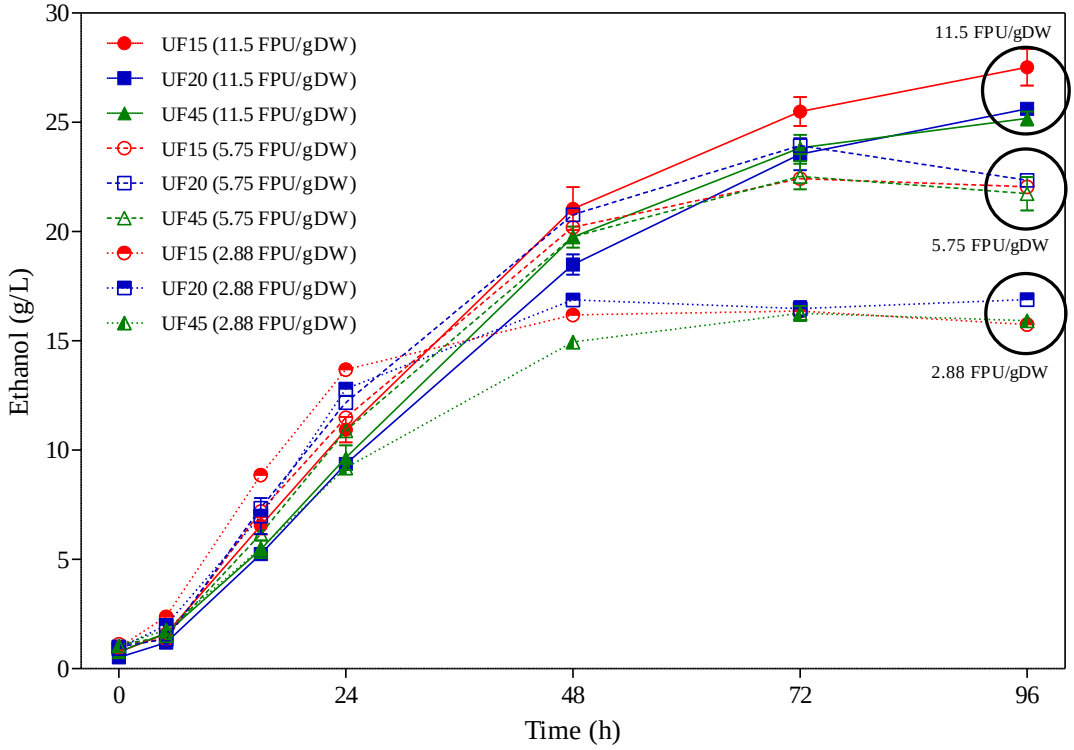

UF15 (11.5 FPU/gDW)
UF20 (11.5 FPU/gDW)
UF45 (11.5 FPU/gDW)
UF15 (5.75 FPU/gDW)
UF20 (5.75 FPU/gDW)
UF45 (5.75 FPU/gDW)
UF15 (2.88 FPU/gDW)
UF20 (2.88 FPU/gDW)
UF45 (2.88 FPU/gDW)
11.5 FPU/gDW
5.75 FPU/gDW
2.88 FPU/gDW
Ethanol (g/L)
Time (h)
0
5
10
15
20
25
30
0
24
48
72
96

Figure 4.

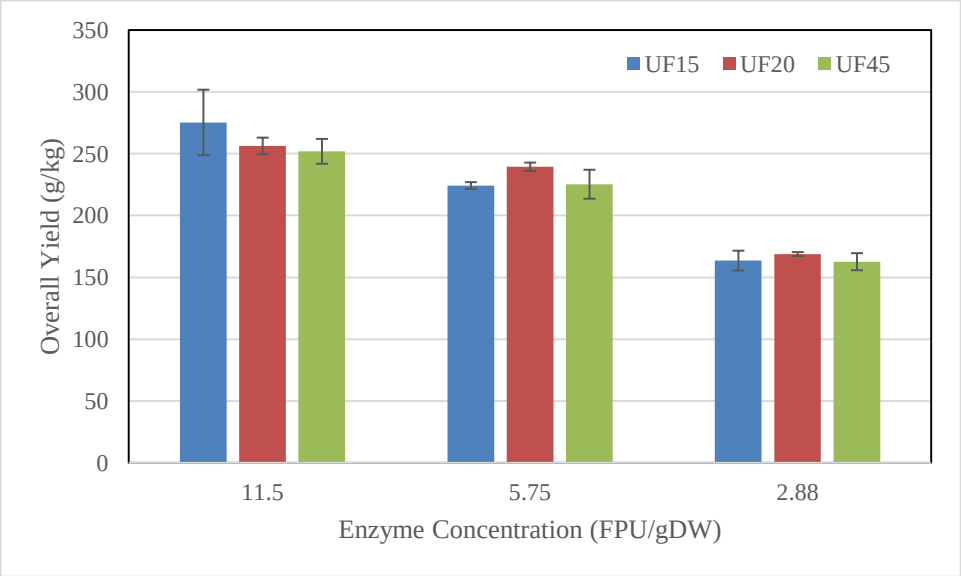

350
300
250
200
150
100
50
0
Overall Yield (g/kg)
UF15
UF20
UF45
11.5
5.75
2.88
Enzyme Concentration (FPU/gDW)

Figure 5.

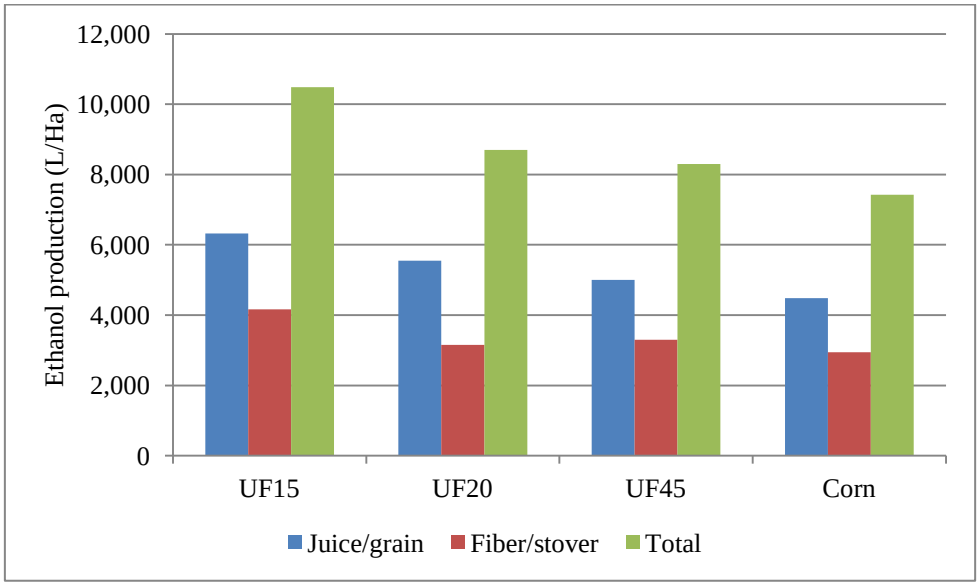

12,000
10,000
8,000
6,000
4,000
2,000
0
Ethanol production (L/Ha)
UF15
UF20
UF45
Corn
Juice/grain
Fiber/stover
Total

Figure 6.