ABSTRACT

BRASWELL, JR., BRANTLY BRODT. Enzymatic Hydrolysis of Whole Stalk Forage Chopped Sorghums for Bio-based Products. (Under the direction of Mari Chinn, Ph.D.).

Sorghum is an annual, low risk crop that can be worked into existing crop rotations across multiple climatic regions. Research into sorghums as bioenergy and bio-based product feedstocks has garnered interest amongst researchers due to its high soluble sugar content, lignocellulosic polymer and starch-based seedhead. However, rapid spoilage makes effective postharvest handling and storage difficult which has limited its use on industrial scales. Development of processes capable of making use of existing infrastructure without significant losses due to microbial spoilage could make sorghums more useful for large scale applications.

One way to effectively make use of all the material available from sorghums is to forage chop it at harvest and hydrolyze all the material through enzymatic hydrolysis. Although efficient, forage chopping sorghums results in increased soluble sugar losses as compared to whole stalk harvesting. However, storage methods including drying, which inhibits microbial activity through lowering moisture content, and ensiling, which allows lactic acid bacteria to consume soluble sugars and produce lactic acid thereby lowering the pH and limiting microbial activity could help alleviate some of these issue.

Hydrolysis of fresh, dried and ensiled sorghums with additives and preservatives to inhibit microbial growth were investigated in this study. Hydrolysis was efficient for fresh and ensiled sorghums, with 65-68% of the available cellulose being converted to glucose. Conversion of dried material was less efficient with only 40% of the cellulose being converted to glucose during hydrolysis. Additionally, it appeared that pretreatment effects

associated with autoclaving (sterilization) could be offset by increasing enzyme loading. The economic and energy inputs of increasing enzyme loading to a very high level should be analyzed in comparison to pretreatment methods in the future.

Enzymatic Hydrolysis of Whole Stalk Forage Chopped Sorghums for Bio-based Products

by
Brantly Brodt Braswell, Jr.

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APPROVED BY:

_____	_____
Mari Chinn, Ph.D Committee Chair	Jose Bruno-Barcena, Ph.D

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Matthew Poore, Ph.D

DEDICATION

This work is dedicated to all my friends and family that helped me along the way.

BIOGRAPHY

Brantly Braswell was born on September 2nd, 1989 in Pinehurst, NC. Born and raised in Fuquay Varina, NC by a large, unique family he grew up loving John Deere tractors, farming, his grandmothers' tomato biscuits and Duke basketball. Following high school at Cardinal Gibbons in Raleigh, NC he went to NC State University to get a degree in Biological and Agricultural Engineering. During his time as an undergrad he met Dr. Mari Chinn and began working with her research team. Upon completion of his undergrad coursework he became a graduate student working for Dr. Chinn.

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1.1 Sorghums for Bioenergy and Bio-based Products

1.1.1 Introduction

As global populations continue to rise, the development of sustainable alternatives to fossil fuels for energy and value-added product production has become critical to meet the growing global demands of a bio-based economy. Research into biomass crops for energy and bio-based products are typically divided into three main categories which are sugar-based, starch-based, and cellulose-based. Starch and cellulose feedstock materials are more widely available than sugar crops but require additional processing steps prior to value added product conversion (Mussatto et al., 2010). Traditional interest in sugar crops such as sweet sorghum, sugarcane and sugar beets has traditionally been focused on the large amount of sugars common to each crop that can be processed for conversion to table sugar or molasses, however, growing energy demand has made these crops garner interest amongst researchers interested in renewable energy processes (Failyer and Willard 1892; Veal et al., 2011).

Similar to sugarcane, sorghum contains sugar rich juice in its stalk, starch rich grains in its seedhead and lignocellulosic polymers that can be used in value added bio-based conversion processes. Although sugarcane may have higher sugar content than sorghum, sorghum has shown the ability to grow across multiple regions on marginal soils that sugarcanes would not be able to. Sorghum has been categorized into four main types: grain, forage, sweet and biomass. Although all types of sorghum can be used for cellulosic biofuel

and bioproduct production, grain and forage sorghums are typically grown for use as animal fodder because of their nutritional characteristics (Zegada-Lizarazu and Monti 2012). Sweet and biomass sorghums are ideally suited for use as a dedicated bioenergy crop rather than animal fodder because of the thick, sugar rich stalk that can grow from eight to twenty feet tall producing a high fermentable sugar content and biomass yield (Whitfield et al., 2012). Research into growth requirements of sweet sorghum (Mastrorilli et al., 1999; Massacci et al., 1996; Woods, 2001) have shown favorable agronomic traits that make it well suited for use as a dedicated biomass crop. The cultivation and practices described for sweet sorghum varieties can be directly applied to forage and biomass sorghums as well (Veal et al., 2011).

1.1.2 Crop Characteristics and Agronomics

Sorghum is a row crop that tolerates multiple soil types with pH ranging from 5.0-8.5 (Zegada-Lizarazu and Monti, 2012) and has been shown to mature in 90 to 180 days (Bennett and Anex, 2009) allowing at least one, or in more temperate regions, multiple crops to be harvested from the same root each year. Planting density varies depending on cultivar and soil type but is typically between 12 and 20 plants per square meter with row width ranging from 35 to 105 cm, although narrower row widths in this range have shown higher sugar and biomass yields (Broadhead and Freeman, 1980). Planting date does not seem to affect sugar concentration, however, a later planting date does reduce overall biomass and total sugar yield (Erickson et al., 2011). Sugar concentration variations based on growth manipulations, more specifically deheading, can result in increased glucose, sucrose and fructose concentrations for certain varieties (Broadhead, 1973; McBee et al., 1983). Harvest for maximum sugar concentration appears to be independent of planting date and is more

strongly related to time after anthesis (Tsuchihashi and Goto, 2004) with harvest ideally occurring from three to five weeks after anthesis depending on variety (Davila-Gomez et al., 2011).

Multiple studies have been done on the relationship between fermentable sugar production and nutrient and water inputs. While it has been reported that application rates around 100 kg N/ha increase biomass yields, it should be noted that higher nitrogen application rates than this appear to decrease the sugar concentration of the juice slightly (Weidenfield, 1984). Turgut et al. (2005) and Geng et al. (1989) reported that the ideal application rate was 100 kg N/ha, while Buxton et al (1999) reported a maximum application rate between 70 and 140 kg N/ha. A study done by Almodares and Darany (2006) indicated that the growth phase of sweet sorghum may have more of an effect on nitrogen uptake than application rate with fertilization during the vegetative stage of growth having a larger impact on stem diameter, dry matter yield and plant height than during later growth phases.

Due to the short time to maturity common with sorghum varieties, use of sorghums in crop rotations could allow growers interested in bio-based markets the opportunity to grow sorghums while maintaining traditional crop production. Furthermore, incorporating sorghums into traditional crop rotations may have favorable effects on the traditional crops grown by farmers. Einhellig and Rasmussen (1989) showed weed growth in years following sorghum crops was significantly less than in years following soybean and corn crops in no till agricultural operations. In addition to weed suppression (Mastrorilli et al., 1999), Patterson and Varvel (1989) showed increased nitrogen uptake in soybean crops following sorghums resulting in higher yields when compared to soybeans in monoculture or soybeans

following corn. Sorghums used in crop rotations were also shown to be effective in limiting plant-parasitic nematodes in corn, soybean and peanut rotations, although total effect on nematode population was cultivar dependent (Gallaher et al., 1991; McSorley and Gallaher, 1993; Rodriguez-Kabana et al., 1990). In addition to the positive benefits of sorghums on crops following them in rotations, use of legumes as a rotation crop with sorghums result in higher grain, biomass and sugar yields because of the nitrogen fixing properties that legumes possess (Blevins et al., 1990; Wortmann et al., 2007).

It is well known that sweet sorghum performs well under drought conditions in comparison to other crops (Woods, 2001) but drought during certain stages of the growing process appear to have more profound effects than others (Mastrorilli et al., 1995). More specifically, Mastrorelli et al (1995) indicated that stress during the leaf stage (40-60 days after planting) resulted in 5 Mg/ha less dry biomass, and severe stress during this stage resulted in 10 Mg/ha less dry biomass, however, drought after anthesis showed no significant change in biomass yields. Studies have investigated the effect of water availability in the range of 2.3 mm to 17.1 mm per day (Yosef et al., 2009), (Curt et al., 1995) with results showing that increased water availability does increase sugar content up to a certain point, typically ranging from . Beyond that point, which varies depending on variety, sugar content remains relatively constant (Whitfield et al., 2012). As a result of its relatively low input and water requirements sweet sorghum is ideally suited for use on marginal soils across many regions of the continental United States with no significant differences in sucrose, glucose, or fructose levels (Smith et al., 1987).

1.1.3 Fermentable Sugars

Research on sweet sorghum juice for direct ethanol fermentation by researchers in the food and bioenergy fields have reported that 20-50% of the plant dry weight is fermentable sugar (Nain et al., 1992; Dolciotti et al., 1998). Fresh sorghum juice typically contains 16-18% fermentable sugar by volume (Wu et al., 2010) with sugar compositions containing 35-77% w/w sucrose, 8-24% w/w glucose and 0-26% w/w fructose and is found primarily in the plants stalk (Bridgers et al., 2011; Liang et al., 2010; Billa, et al., 1997; Sipos, et al., 2009). Harvest date effects sorghum juice composition with Sipos et al. (2009) reporting that harvest prior to the reproductive growth stage results in low total sugar concentrations comprised largely of fructose, 65% w/w of the total sugar concentration. Sucrose formation begins only after the reproductive phase of ontogenesis, especially after the heading stage (McBee and Miller, 1982), with the internodes of the plants stems ceasing to elongate and becoming storage sinks for accumulated sucrose (Hoffman-Thoma et al., 1996). As sucrose accumulation occurs during plant maturation, it is typically at the expense of glucose and fructose (Almodares et al., 2007). Broadhead (1972) reported that total sugar yield was not effected by harvest date after maturity but total sugar concentration increased. This is likely a result of a decrease in moisture content of the plant thereby reducing juice volume without reducing the amount of sugars in the juice leading to an increase in sugar concentration. Within the stem itself, Billa et al (1997) examined moisture content and soluble sugar composition and indicated that the pith fraction of the stalks contain higher moisture, sucrose, and glucose levels than the rind fraction.

Although the fermentable sugar content of sorghum was less than that of other sugar crops such as sugarcane and sugar beets, sorghums have the ability to grow in more temperate areas and produce starch in the seedhead and lignocelluolosic polymers that can be converted into fermentables (de Vries et al., 2010).

1.1.4 Lignocellulosic Bagasse

A major advantage of sweet sorghum as a bioenergy feedstock is the fact that it contains not only fermentable sugars and starches, but also lignocellulosic bagasse that can be utilized. Traditionally, the bagasse has been studied as a potential animal feedstock due to the bagasse having fodder qualities that do not require chemical or physical upgrading prior to incorporation into animal feed (Blümmel et al., 2009). However, to improve the usefulness of sweet sorghum as a bioenergy and bio-based product feedstock, the conversion of the 3.9-4.5 megagrams per hectare of lignocellulosic bagasse is being considered (Blümmel et al., 2009).

A study done by Reddy and Yang (2007), showed that sorghum leaves and stems have approximately 65% cellulose on a dry weight basis, while containing relatively low lignin contents of only 6.5-9% on a dry weight basis. Another study found cellulose levels of depithed and extracted bagasse to be between 42 and 48% on a dry weight basis (Beleyachi and Delmas, 1995). As reported by Whitfield et al (2014) bias in the commonly used NREL composition analysis procedure may result in lower cellulose values due to losses of the pith during analysis. Considering the high biomass yield and large amounts of cellulose available in the leaves and stems of sweet sorghum, accessing these cellulosic materials during conversion for additional fermentable sugars would increase overall conversion yields.

Parrish et al (1985) and Yosef et al (2009) reported that grains generated from sweet sorghum production produced 1.99-5.17 Mg/Ha dry biomass with a starch content that ranged from 0.64-1.91 Mg/Ha 40 days after anthesis. Furthermore, Parrish et al (1985) estimated that the whole stem and grain of a sweet sorghum plant would be capable of producing 9.7 Mg/ha of fermentable sugar. Yosef et al (2009) reported 4-11.5 Mg/Ha dry-weight of cellulose and hemicellulose for sorghum stalks and leaves with the stems consisting of 48-52% cellulose, 40-50% hemicellulose and 3-9% lignin and the leaves consisting of 40-49% cellulose, 46-53% hemicellulose and 6-10% lignin.

1.2 Fermentation of Sweet Sorghum Sugars

1.2.1 Fermentation Methods

Fermentation of sweet sorghum has been completed using three different strategies: juice fermentation, submerged solids fermentation, and solid state fermentation. Historically, direct fermentation has been performed on sorghum juice, where pressed juice has been inoculated with fermentative microorganisms, typically bacteria or yeast, and allowed to ferment (Liu and Shen, 2008; Shen et al., 2009; Mei et al., 2009). An orthogonal optimization experiment done by Ronghou and Fei (2008), reported that sweet sorghum juice fermentation was best when temperature was 37˚C, with agitation rate of 200 rpm, and pH 5.0. These conditions are common to other yeast fermentation processes. Juice fermentation has been studied extensively (Bridgers et al., 2011; Wu et al., 2010; Laopaiboon et al., 2007; Liu, et al., 2008) with some studies reporting sugar conversion efficiencies of up to 99.20% (de Mancilha et al., 1984). Interestingly, a comparison of 64 different yeast strains

converting sugars in the same liquid media showed that 20 of the yeast strains had a sugar conversion efficiency of over 90%. Of the 20 yeast strains with higher than 90% conversion efficiency, all 20 were *Sacchromyces*. Although juice fermentations can be very efficient, in some cases greater than 99% sugar conversion, challenges with harvest techniques and low juice press efficiencies limit their use in commercial applications. Commonly, the sorghum stalk is harvested whole and the intact stalks are pressed through a roller mill to extract the juice, with recoveries usually below 50% of the available juice (Monroe et al., 1984; Coble and Reidenbach, 1985; Cundiff, 1992). Due to these low juice press efficiencies, researchers have looked at alternative processes capable of utilizing all of the fermentables present in sorghum biomass.

One approach used to consume all present fermentables in sorghum is submerged solids fermentation where the solids are added into an ongoing juice or defined medium fermentation (Rolz et al., 1979). Kargi et al (1985) and Coble et al (1984) both attempted this process and reported higher ethanol yields when compared to juice fermentation alone. The benefits of this approach, however, seem negligible as Kargi et al (1985) also reported in their work that separate juice and bagasse conversion resulted in no significantly different ethanol yields than converting chopped stalks together. Furthermore, Yu et al (2008) reported that the optimal particle size for the solids added back to the juice fermentation was very small, meaning that additional post-harvest treatment must be done. This added mechanical grinding to reach optimal particle size would increase energy and labor inputs thereby making the conversion process more costly.

Solid-state fermentation, defined by Pandey (1992) as the fermentation of solids in absence of free water, could be a useful conversion process to combat the challenges associated with direct juice and submerged solids fermentation. In solid-state fermentation biomass sorghum is inoculated directly and allowed to ferment in the absence of free flowing water. Furthermore, the naturally high moisture content of the solid substrate are necessary for adequate microorganism growth, with moisture being adsorbed and integrated within the pores of the solid. The solid substrate in solid-state fermentation processes acts as the carbon source and growth matrix for the microorganisms that are being used in the conversion process.

Bryan (1990) looked at using larger sweet sorghum chips (0.6 cm) for ethanol production through solid-state fermentation (0%-0.2% w/w Distillers active dry yeast, 3-4 kg fresh sweet sorghum) in 7-1 insulated fermenters and found that 80% of the theoretical ethanol yield could be achieved. However, Bryan also found that fermentation time was 60-120 hours, which comparatively was much greater than most ethanol fermentations with free-flowing liquid.

1.3 Conversion of Lignocellulosic Biomass

1.3.1 Conversion Overview

In order to access the sugars present in the lignocellulosic biomass fractions found in sorghums, conversion of the complex polymers to simple sugars must be completed. The Process development for converting lignocellulosic biomass to bio-based products has been researched extensively. The reduction of lignocellulosic biomass to simpler saccharides begins with delignification, where lignin is broken down to increase accessibility of cellulose

and hemicellulose complex structure of the lignin-cellulose matrix. Subsequently, the cellulose and hemicellulose are depolymerized to produce free sugars that can then be used in fermentations for a variety of different end-products depending on the microorganisms chosen (Lee, 1997). Due to the amounts of lignin commonly found in lignocellulosic biomass pretreatment processes must be utilized to remove the lignin. Typically, the lignin content of softwoods is 25-35%, hardwoods contain approximately 18-25% lignin, and herbaceous species and agricultural residues range in composition from 10-30% lignin (Lee, 1997; McMillan, 1994; Reshamwala et al., 1995). Typically, pretreatment for lignocellulosic biomass includes mechanical comminuition, alkali swelling, acid hydrolysis, steam and other fiber explosion techniques, exposure to supercritical fluids, or hydrothermal pretreatment (McMillan, 1994). Sorghum varieties generally have lower lignin contents than other lignocellulosic feedstocks, allowing milder pretreatment methods to be used. The potential of using less intense pretreatment methods may results in lower processing costs when considering using sorghums as a biomass feedstock for bioenergy and other bio-based products.

Susceptibility of cellulosic material to enzymatic degradation depends on structural features including: the degree of water swelling, crystallinity, molecular arrangement, lignin content and the capillary structure of cellulose fibers (Fan et al., 1980) which can be altered through pretreatment. Sipos et al (2009), indicated that sweet sorghum bagasse steam pretreated at 190˚C for 10 minutes and 200˚C for 5 minutes resulted in 89% and 92% conversion of the theoretical glucose of the separated, washed fiber fraction. Dilute ammonia pretreatment on crushed, dried stalks with leaves, roots and grains removed (1.5 kg

sorghum fibers, 28% v/v ammonium hydroxide, 1 h at 160ºC and 160 psi) resulted in 44% removal of the original lignin from sorghum fibers and increased glucan digestibility by cellulase enzymes (Spezyme 60 FPU/g glucan, Novozyme 188 64 CBU/g glucan) 46% versus untreated bagasses (84% glucan digestibility pretreated bagasse, 38% untreated bagasse) as a result of increased surface area and porosity (Salvi et al., 2010). Xu et al (2010) investigated using sulfuric acid (0.5% to 1.5% w/w) on ground sorghum reporting that pretreatment increased cellulose from 40.5% d.w. to 92% d.w at 1.0% w/w sulfuric acid. However, at 1.5% w/w sulfuric acid the amount of cellulose measured decreased to 80.7% as a result of degradation.

1.3.2 Enzymatic Hydrolysis

Once lignin fractions have been altered after pretreatment methods, cellulose and hemicellulose are depolymerized through hydrolysis. Commonly, acid, alkaline, hydrothermal and/or enzymatic hydrolysis are used in order to break cellulose and hemicellulose down into their component sugars needed for fermentation (Ye and Jiayang, 2002). Enzymatic hydrolysis has been found to have a lower unit cost than acid or alkaline hydrolysis (Duff and Murphy, 1996) because it is typically completed at mild temperature, pH and pressure conditions. Acid hydrolysis is considered limited in its economic feasibility because of the challenges associated with use of strong acids in subsequent fermentation unit operations, including sugar degradation problems and additional waste disposal concerns. Although concentrated acid hydrolysis processes have been researched and some methods shown relatively minor sugar degradation in combination with cellulose to glucose

conversion yields approaching 100%, waste disposal issues and the high cost of acid consumption limits large scale implementation of this practice (Duff and McMurphy, 1996).

However, generation of inhibitory compounds such as weak acids, furans and phenolic compounds during acid based hydrolysis techniques have been reported (Palmqvist and Hahn-Hagerdal, 2000) leading to inhibition of cellulolytic enzyme activity (Heredia et al., 1990). Although generation of these inhibitory compounds associated with acid based hydrolysis techniques have been observed, phenolic compounds (phenolic acids, flavonoids and tannins) located in pericarp, testa, aleurone layer and endosperm (Dykes and Rooney, 2006) of sorghum can also inhibit enzymatic hydrolysis through protein binding, with reports of tannins being able to bind 12 times their weight of protein through hydrogen bonding. Although tannins protect sorghum grains against insects, birds and fungal attack, their presence resulting from a particular biomass feedstock converted by enzymatic hydrolysis systems should be limited because of their ability to bind proteins (Duodu et al., 2003). Phenolic compounds are present in all sorghum varieties creating potential challenges to biochemical conversion approaches for processing, but genetic differences in the plant result in varying colors and thickness of the pericarp. Sorghums are divided into three groups based on their genetics and chemical analyses with group I sorghums lacking a pigmented testa layer and having no tannins, group II sorghums have a pigmented testa layer that contains condensed tannins that can be extracted with acidified methanol (1% HCl) and group III sorghums have a pigmented testa layer and a dominant S gene generating polyphenols in the testa layer and the pericarp with tannins extracted with either methanol or acidified methanol (Rooney et al., 1982). Group I sorghums (white sorghums) are likely to

result in more efficient hydrolyses by enzymatic operations due to their lower concentration of phenols and the fact that they do not contain tannins lending them more favorably to use in bio-based conversion processes.

Enzymatic hydrolysis of cellulose in lignocellulosic material utilizes the synergism between cellobiohydrolases, endogluconases and β-glucosidases. There are several steps to the enzymatic cellulose hydrolysis process which starts with the migration of enzymes from the bulk aqueous phase to the surface of the cellulose particles with the enzymes being adsorbed to the surface of the cellulose particles creating enzyme-substrate complexes. Cellobiohydrolases (CBH) or exogluconases and endogluconases (EG) start to break down the long cellulose chains with the CBH enzymes removing cellobiose units from the reduced ends of cellulose chains and EG attacking regions of low crystallinity randomly within the cellulose fiber creating shorter chains. The resulting products of the synergism between endogluconases and cellobiohydrolases are cellobiose, glucose and cellodextrins in the aqueous phase. β-glucosidases, if available, hydrolyze cellobiose to glucose which completes the cellulose to glucose hydrolysis process as well as relieves end product inhibition for the CBH enzymes (Jalak et al., 2012; Reese et al., 1950; Wood and McCrae, 1979; Ladisch et al., 1981; Coughlan and Ljungdahl, 1988). Numerous factors play a role in the effectiveness of cellulose conversion by cellulase enzymes including the substrate structure and concentration, source and activity of the enzymes, concentration of end products, temperature, pH and concentration of other compounds in the hydrolysis environment (Coughlan, 1992).

Use of accessory, such as hemicellulase enzymes, to work synergistically with cellulase enzymes to increase degradation of biomass polysaccharides from corn stover and subsequently increase sugar production has been reported (Selig, et al., 2008) (Kumar and Wyman, 2009). Hemicellulose is more accessible to enzymatic hydrolysis than cellulose because it does not form tightly packed crystalline structures like cellulose. However, hemicellulose is a more complex structure than cellulose requiring several different enzymes with specific roles for complete hydrolysis (Gilbert and Hazelwood, 1993). The required enzymes for hydrolysis of hemicellulose are: endo-β-1,4-xylanase, β-xylosidase α-L-arabinofuranosidase, α-glucoronidase, acetylxylan esterase, ferulic acid esterase and *p*-coumaric acid esterase. The endo-xylanase attacks the main of xylans and β-xylosidase hydrolyzes xylooligosaccharides to xylose. The α- arabinofuranosidase and α-glucoronidase remove the arabinose and 4-*O*-methyl glucouronic acid substituents from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid or between arabinose side chain residues and phenolic acids such as ferulic acid and *p*-coumaric acid (Saha, 2003).

Although enzymatic hydrolysis of lignocellulose can be effective at breaking down the long cellulose and hemicellulose chains into easily utilized monosaccharides, these enzymes can prove difficult to produce on a commercial scale with high activities that are cost effective. In addition, enzyme activity and function are susceptible to inhibition, including end-product inhibition during hydrolysis (Duff and Murphy, 1996). A wide range of organisms including aerobic, anaerobic, mesophilic and thermophilic bacteria and fungi can produce cellulase enzymes capable of hydrolyzing lignocellulosic material (Bisaria,

1991). Despite the ability of cellulolytic bacteria, specifically *Clostridium thermocellum* and *Bacteroides cellulosolvens*, to produce highly active cellulase enzymes, they do not produce large enough quantities that are also extracellular to make them commercially viable. Most commercially available cellulase enzymes are produced by fungi (Duff and McMurray, 1996).

Combining certain pretreatment methods, which can alter the physical characteristics of the biomass, with enzymatic hydrolysis can help reduce some of the inhibition problems common to enzymatic hydrolysis. Commonly, steam pretreatment with an acid catalyst is used to degrade lignin, which, when coupled with enzymatic hydrolysis was shown to be efficient in hydrolyzing glucose from corn stover, with yields of 87-100% of the available glucose being reported (Ohgren et al., 2007). Matsakas and Christokopoulos (2013) were able to convert 60% of the cellulose through enzymatic hydrolysis of hydrothermally pretreated washed, milled sorghum bagasse. Rohowsky et al (2013) also investigated using hydrothermal pretreatment prior to enzymatic hydrolysis of pressed forage chopped sorghum and reported cellulose to glucose conversion efficiencies of 60%.

1.4 Ensiling

1.4.1 Overview

Despite the ability of many non-grain sorghum cultivars to grow to maturity in a short 90 to 180 day period, with low fertilizer and water input requirements, challenges associated with harvesting and storage have limited its use as a bioenergy feedstock (Smith et al., 1987). One major challenge associated with effectively storing sweet sorghum is its susceptibility to microbial contamination as a result of its high sugar content, high moisture content and ideal

pH for growth. Due to these characteristics, microbial proliferation can result in significant sugar losses, with up to 50% of the free sugars being lost during the first 24 hours after harvest (Miron et al., 2005). Furthermore, the seasonal availability of sorghums creates challenges in using infrastructure efficiently and scheduling labor. According to Bennett and Anex (2009), capital costs of biorefineries capable of converting large amounts of sweet sorghum generated during harvest periods are not economically feasible as they would sit dormant for 6-8 months out of the year when freshly harvested material is unavailable. Aside from diversifying the feedstocks entering a given biorefinery facility throughout the year, use of these facilities year-round for economic viability will require development of better storage methods that preserve structural carbohydrates, especially in sorghums.

1.4.2 Process Characteristics

Ensiling has emerged as a potential solution to the problems associated with long term storage of sorghums that can be applied specifically to forage, sweet and biomass sorghums for development of bio-based products. Typically green biomass storage is done through two methods: (1) Dropping the moisture level to approximately 30% through wilting in the field or drying, or (2) Allowing an acidic fermentation to take place in order to inhibit clostridial activity and spoilage (Whittenbury et al., 1967). As stated by Philipp et al (2007), the goal of ensilage is to prevent deterioration of plant materials through lactic acid fermentation under anaerobic conditions. Prior to the ensiling process lactic acid bacteria, enterobacteria, yeasts, molds, clostridia, bacilli, acetic acid bacteria and propionic acid bacteria are commonly found on plants. Of these typical microorganisms commonly found on plants prior to ensiling, lactic acid bacteria and enterobacteria account for most of the

microbial population (Pahlow et al., 2003). During the ensiling process, lactic acid forming bacteria consume sugars and produce a mixture of acetic acid and lactic acid under limited oxygen conditions increases the hydrogen ion concentration lowering the pH to inhibit additional microbial activity (Whittenbury et al., 1967). In the context of bioenergy production, lactic acid production results in less readily available sugars for ethanol fermentation because they have been consumed during the ensiling process. Free sugar losses in the ensiling process are substantial, with losses up to half of the available sugars, and variations between varieties have been reported (Miron et al., 2005). However, once the pH drops as a result of microbial activity on those sugars the additional lignocellulosic components can be preserved. Attempts have been made to modify the ensiling process in such a manner that fermentable carbohydrates remain at higher levels during extended storage periods (Schmidt et al., 1997; Tengerdy, et al., 1996). Yet, depending on the conversion process used, reduction in the soluble sugar content may be beneficial as high initial sugar concentrations can inhibit some processing steps, including enzymatic hydrolysis.

1.4.3 Fermentable Sugar Losses

According to Pahlow et al (2003) there are 4 main stages during the ensiling process, however, the last stage discussed in their work is related to surface decay of forage material exposed to air after ensiling has been completed and is not applicable to the context of this review. The first phase is the initial aerobic phase in which the effect of atmospheric oxygen diminishes. While oxygen is still trapped in the forage matrix, plant and microbial respiration take place generating heat, plant enzymes remain active and proteases begin

decomposing proteins to amino acids. Once all the oxygen has been consumed, typically within a few hours, through cellular respiration, the second phase or "main fermentation stage" begins. During the main fermentation stage, which ranges from 1 week to 1 month depending on ensiling conditions and crop properties, the microbial population transitions from a mixed community of enterobacteria, clostridia, bacilli and yeast to predominantly lactic acid bacteria populations. This results in fermentable sugar losses due to lactic acid fermentation. However, for use as a lignocellulosic feedstock, the high initial sugar concentration common in sorghums may allow full ensiling to occur without effecting the composition of the structural material. After the "main fermentation phase" is complete the "stable phase" is said to begin and relatively little changes in terms of material composition occur if the ensiled material is not exposed to air. Sun et al (2010) successfully ensiled sorghum bagasse while only removing 20% of the juice for fermentation prior to beginning ensiling, indicating that complete ensilage can occur without utilizing all the readily available sugars during the process. Complete ensilage without utilization of structural carbohydrates or all of the readily available sugars could preservation of the structural carbohydrates in sorghum bagasse for conversion to biobased products. In addition to preserving readily available sugars and structural carbohydrates, ensiling could also be considered a type of mild pretreatment because the acidic conditions after ensiling can improve the effectiveness of enzymatic hydrolysis following ensiling (Linden et al., 1987).

1.4.4 Stabilization

In order to successfully supply harvested sorghum biomass on a year round basis, storage processes capable of handling the highly perishable sorghum rapidly and effectively

must be used. Some of the storage methods, other than ensiling, proposed in the literature include cool/cold storage of whole stalks, drying of whole stalks, enzyme assisted ensiling, sulfur dioxide preservation of forage chopped material, evaporating the juice to 60% syrup and ensiling the chopped stalks in 0.5% formic acid. (Schmidt et al., 1997; Sipos et al., 2008). Attempts to store whole stalks in cool/cold storage was attempted by Cundiff and Parish (1983) and was successful in maintaining whole stalks for up to 150 days. However, energy inputs, material handling and high upfront costs for cooling rooms capable of holding large quantities of whole sweet sorghum stalks are not economically feasible (Bennett and Anex, 2009). In a separate study done by Parrish and Cundiff (1985) drying whole stalks as a storage method was investigated and shown to adequately store sweet sorghum but economic and energy input costs associated with this method were not feasible on an industrial scale as well..

Schmidt et al (1997) used formic acid to create ensiling conditions without utilizing large amounts of sugars from the biomass itself. They were able to not only successfully maintain sugar levels using this approach but also slightly increase reducing sugar yields. It is likely that the slight increase in reducing sugar levels over the 60 day treatment period is a result of partial hydrolysis of the starch fraction of the biomass.

1.5 References

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Chapter 2 Enzymatic Hydrolysis of Fresh and Dried Chopped Sorghum

2.1 Introduction

Sorghums (sweet, forage and biomass types) are attractive feedstocks for bioconversion to biofuels and biobased products because of their high source of fermentables in the form of directly fermentable soluble sugars and structural carbohydrates. Favorable agronomic features including, low growth input requirements, quick growth period and ability to grow across multiple regions of the United States as an annual low risk crop also make sorghums appealing as a biomass feedstock. Sorghum crops have been reported to yield between 6 and 16 dry Tons/ Ha. The soluble sugar fractions make up 20 to 50% of the plants biomass on a dry-weight basis (Nain et al, 1992), thus the remaining lignocellulosic and starch fractions of the plant can provide more material for fermentable sugars. Historically, sorghum feedstocks have been pressed for juice with leaves and seedheads removed for use in making molasses and demonstrations of direct ethanol fermentation as interest in biofuels has increased (Veal et al., 2011). The remaining bagasse after pressing has commonly been used as animal fodder (Zegada-Lizarazu and Monti, 2012). Although the fermentation of sweet sorghum juice can be efficient with reports of up to 99% of theoretical ethanol yield being achieved (de Mancilha et al., 1984), the manual labor involved in preparing sorghum stalks, if harvested as cut plants, and juice press efficiencies regardless of harvest approach (cut stalks, chopped material) present limitations in processing logistics and crop utilization (e.g. processing rates, equipment needs, storage, feedstock quality).

Despite suggestions for improvements in juice press efficiencies (Cundiff, 1992), (Moroe et al., 1984) the high concentrations of sugars in the juice and natural pH, ranging from 4.4-5.6, make sorghum juice an ideal environment for fermentative microorganisms, including contaminant species (Laopaiboon et al., 2009; Davila-Gomez et al., 2011; Chohnan et al., 2011). In addition to challenges with minimizing juice deterioration (i.e. sugar losses) from spoilage, difficulties associated with storage and handling of large volumes of juice also limit its use in commercial applications.

Rather than only pressing sorghum for its juice and using the remaining solids (bagasse) as animal fodder, use of the lignocellulosic bagasse has garnered interest amongst researchers for use as a bioenergy and bio-based feedstock.

Previous researchers have ground pressed bagasse and fermented the material through submerged solids fermentation where solids are added back into an ongoing juice fermentation or have used the pressed bagasse alone in solid state fermentation systems for ethanol production (Rolz et al., 1979; Ballesteros et al., 2004). Submerged solids fermentation of the juice and bagasse along with separate juice and bagasse fermentation can generate higher ethanol yields than juice fermentation alone (Coble et al., 1984; Kargi et al., 1985). However, very small optimal particles sizes proposed for these fermentation methods (Yu et al., 2008) and lack of significantly higher ethanol production compared to direct fermentation of unfractionated sorghum stalks (Kargi et al., 1985) suggest that the additional unit operations may not be cost-effective for processing sorghums for bioenergy and biobased products.

Table 2-1: Composition of Lignocellulosic Materials in Percent Dry Weight

Lignocellulosic Material	Cellulose (%)	Hemicelullose (%)	Lignin (%)	Source
Hardwood Stems	40-55	24-40	18-25	Zhu and Pan (2010)
Softwood Stems	45-50	23-35	25-35	Galbe and Zacchi (2007), Zhu and Pan (2010)
Wheat Straw	30-40	20-25	15-20	Zhu and Pan (2010), Talebnia et al (2010)
Coastal Bermuda Grass	25-32	25-35	6-20	Jung et al (2009), Reshamwala et al (1995)
Switch Grass	31-45	25-33	12-19	Cantrell et al (2009), Reshamwala et al (1995)
Corn Stover	30-38	22-30	18-23	Galbe and Zacchi (2007), Zhu and Pan (2010), Cheng et al (2009)
Sugarcane Bagasse	31-50	23-25	22-25	Pandey et al (2000), Das et al (2004), Pessoa Jr. et al (1997)
Sorghum Bagasse	41-48	18-26	8-18	Ghoshadrou et al (2011), Khristova and Gabir (1990), Gobin et al (2010)

Separation of juice and bagasse requires additional energy, labor and infrastructure aside from the added processing steps for conversion operations. Development of a whole stalk conversion process that takes advantage of all the biomass available from sorghum in fewer unit operations for fermentable sugars has the potential to mitigate many of the challenges described with harvest and pre-processing operations.

One way to process whole sorghum stalks and minimize harvest labor and increase efficiency is through forage chopping (Veal et al., 2011). However, this approach can increase material spoilage rates post-harvest by natural microflora, 50% of fermentable soluble sugars within 24 hours after harvest (Veal, 2009) suggesting that material will need

to be processed quickly (e.g. in-time harvest/use system) and additional processing treatments may be necessary to minimize spoilage.

As is the case with any lignocellulosic material selected for conversion, pretreatment is a necessary step to disrupt the lignocellulosic matrix, making cellulose more accessible to enzymes that can convert the carbohydrate polymers to fermentable sugars. However, pretreatment is one of the most expensive steps in the lignocellulosic material to biofuel process (Mosier et al., 2005). Typical pretreatment methods include mechanical comminuition, alkali swelling, acid hydrolysis, steam and other fiber explosion techniques, liquid hot water and exposure to supercritical fluids (McMillan, 1994). As a result of its lower lignin content relative to other lignocellulosic materials, as seen in Table 2, harsh pretreatment methods may not be necessary for sorghums, potentially reducing the number of unit operations and associated processing costs.

Objectives

The overall goal of this work was to evaluate the potential of converting whole sorghum stalks as chopped biomass feedstock to fermentable sugars that can be used for the production of biofuels and biobased products. Objectives were to: (1) Evaluate the effects of enzyme loading rates on hydrolysis of whole sorghum stalks for the release of fermentable sugars over time and (2) Study the usefulness of additives in preventing loss of hydrolysis sugars byproduct formation by natural microflora for dried and fresh preparations of sorghum. Sterilization (autoclaving) as a preparation of whole sorghum stalks was completed for each treatment combination to establish a baseline for release of fermentable sugars. It was assumed that this approach would minimize the effects of natural microflora.

2.2 Materials and Methods

2.2.1 Sorghum Feedstocks and Initial Composition Analysis

Sorghum (*Sorghum bicolor*) cultivars Sugar T and AF-7401 were harvested during the 2010 and 2014 cropping seasons, respectively. Both varieties were forage chopped, stored in vacuum sealed bags on ice (0.5 inch) and processed within 24 hours after harvest. Sugar T was oven dried (45ºC) immediately after harvest and stored in plastic bags at indoor ambient conditions in a dark area prior to use as a dried sorghum material in enzymatic hydrolysis experiments. AF-7401 was taken immediately from the field, weighed and used as fresh material for enzymatic hydrolysis experiments. Sorghum composition analysis was completed using a modified NREL procedure developed by Whitfield et al (2014).

2.2.2 Experimental Design

Enzymatic hydrolysis experiments were completed on fresh and dried forage chopped sorghum. The effects of cellulase (CTec2 10 FPU/g, CTec2 25 FPU/g, CTec2 50 FPU/g) and hemicellulase (0%, 1.5%, 3% w/w) enzyme loading levels and hydrolysis time (0, 12, 24, 36, 48,and 72 hours) were investigated for fresh and dried forage chopped sorghum prepared as sterilized material (autoclaved) and with additives to inhibit naturally occurring microflora, 1) sodium azide (SA), 2) lysozyme and nisin (LN) and 3) sodium azide, lysozyme and nisin (LNSA) combinations. Each treatment combination and controls (no enzyme) were completed in triplicate and were destructively sampled. Response variables were measured over time and included glucose, sucrose, fructose, cellobiose, xylose, arabinose, lactate, acetate and ethanol concentrations. Analysis of variance for main and interaction effects and pairwise comparisons between preparations within each sorghum cultivar material were

evaluated in SAS. Multiple linear regression using Proc GLM was used to determine effects of enzyme (CTec2, HTec2) loading levels, material treatment and time had on sugar and byproduct formation versus controls. To determine differences between treatment groups, controls were removed from analysis and Proc GLM was run on CTec2, HTec2, treatment and time to determine statistical significance.

2.2.3 Enzymes and Chemical Additives

Table 2-2: Enzyme and Chemical Additive Stock Solutions Added to Treatments

Name	Composition	Applicable Treatments
Buffer A	10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl	Autoclaved, LN
Buffer B	10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl 0.02% w/v Sodium Azide (20 mg/L)	SA, LNSA
CTec2 Stock	40 ml Buffer A or Buffer B, 4 ml CTec2 (145 FPU/ml)	Buffer A- Autoclaved, LN Buffer B- SA, LNSA
HTec2 Stock	39 ml Buffer A or Buffer B, 1 ml HTec2 (90 IU/ml)	Buffer A- Autoclaved, LN Buffer B- SA, LNSA
Lysozyme Nisin Stock	100 ml Buffer A or Buffer B, 1125 mg/L Lysozyme, 375 mg/L Nisin	Buffer A- LN Buffer B- SA, LNSA

Two different buffers were prepared for sodium azide and non-sodium azide treatments, respectively, to apply the multiple additive levels to the experimental units. Buffer A (10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl) was used for all treatments that did not contain sodium azide and buffer B (10.7 g/l sodium citrate (0.05 M),

0.02% w/v Sodium Azide (20 mg/l) adjusted to pH 5.0 using HCl) was used for all treatments that did contain sodium azide. Enzyme stock solutions were made such that 1 ml of CTec2 enzyme stock would be the equivalent of 10 FPU/g material (40 ml Stock A or Stock B depending on treatment, 4 ml CTec2). The activity of CTec2 was 145 FPU/ml as determined using Filter paper assay (Adney and Baker, 1996) and 1 ml of HTec2 stock solution (39 ml Stock A or Stock B depending on treatment, 1 ml HTec2). The HTec2 activity was 90 units/ml as determined using a xylanase activity assay. For treatments containing lysozyme and nisin, a stock solution was made such that 1 ml of stock solution would provide 112.5 µg/ml of lysozyme and 37.5 µg/ml of nisin (112.5 mg lysozyme and 37.5 mg of nisin, 100 ml Stock A or Stock B depending on treatment). Lysozyme, nisin and sodium azide were selected as additives for treatments to minimize the influence of gram-positive bacteria (e.g. Lactic acid bacteria, clostridia, bacilli) and fungi (Chung and Hancock, 2000; Ruhr and Sahl, 1985; Lichstein and Soule, 1943).

Filter paper assays were completed based on the filter paper assay (Adney and Baker, 1996) protocol with the described concentrations of Lysozyme, Nisin, Sodium Azide mentioned previously and the acetate and lactate concentrations of samples with high byproduct concentrations in the hydrolysate.

Table 2-3: Cellulase activity in the presence of additives, additive combinations and common byproducts

Additive	FPU/ml
Lysozyme	181
Nisin	200
SA	201
Acetate	194
Lactate	138
LN	210
LNSA	192
Control	204

2.2.4 Enzymatic Hydrolysis

Fresh and dried chopped sorghum material (1.25 g as it was) was loaded into Falcon tubes (50 ml) to achieve a final loading of 6.25% w/v in hydrolysis treatments (1.25 g fresh AF7401 material, 65% moisture content, 0.438 g dry; 1.25 g Sugar T, 6% moisture, 1.18 g dry). For sterilized treatments, biomass was autoclaved (121 C, 19 psi) as allocated in Falcon tubes for 60 minutes on a liquid cycle. For non-sterilized treatments, Falcons tubes were exposed to UV for 1 hour in a biosafety cabinet prior to addition of enzymes and additives. For dried Sugar T, 1 ml, 2.5 mls and 5 mls of CTec2 (12.5 FPU/ml) was added to achieve 10 FPU/g material, 25 FPU/g material, 50 FPU/g material, respectively and 0 ml, 0.5 mls, and 1 ml of HTec2 was added to achieve per g of material, respectively in the appropriate Falcon tubes. For fresh AF7401 1 ml, 2.5 mls and 5 mls of CTec2 (145 FPU/ml) was added to the appropriate Falcon tubes to achieve 10 FPU/g material, 25 FPU/g material, 50 FPU/g material, respectively. The effects of lysozyme nisin treatment was not studied in fresh material due to lack of resources needed to process fresh material in a timely manner. For treatments that received additives, 13-18 ml by increments of 0.5 ml of buffer B (0.05 M Sodium Citrate buffer x 20 mg/L sodium azide, pH 5) were added to sodium azide

treatments, 13-18 ml by increments of 0.5 ml of buffer A (0.05M, pH 5) were added to each falcon tube to make up to 20 ml. Falcon tubes were incubated in a shaking water bath at (50ºC, 50 RPM) and destructively sampled at times (12, 24, 36, 48 and 72 hours). Time zero samples were sampled immediately after the addition of enzymes and additives (without incubation). Samples were processed by centrifugation (2731 x g, 10 min) and hydrolysate aliquots (2 ml) were removed and stored at -80C for HPLC analysis.

2.2.5 HPLC Analysis

The response variables glucose, sucrose, fructose, xylose, cellobiose, arabinose, lactate, acetate and ethanol were determined using high performance liquid chromatography (HPLC). HPLC samples were thawed, centrifuged (14908 x g, 10 min) and filtered through 0.2µ Whatman (Maidstone, UK) syringe filters prior to analysis. A Phenomenex (Torrance, CA) Rezex ROA column (300mm x 7.8 mm) at 55ºC in 50-minute runs with 0.6 mL/min HPLC water (Sigma-Aldrich) containing 5 mM sulfuric acid as the eluent was used to measure glucose, xylose, arabinose, cellobiose, acetic acid and lactic acid concentrations by refractive index detection (RID) using. A Phenomenex RPM column (300mm x 7.8 mm) at 85 ºC using HPLC water (Sigma, Chromasolv) as the eluent (0.6 ml/min) was used to measure glucose, fructose and sucrose concentrations by RID. This procedure was similar to the one used by Whitfield et al (2014).

2.3 Results and Discussion

2.3.1 Composition Analysis

Dried sorghum cultivar Sugar T contained 38.6% cellulose, 31.5% hemicellulose, 19% extractives and 12.6% acid insoluble lignin (AIL) on a dry weight basis with 6.5%

moisture content at time of use. Fresh sorghum cultivar AF7401 contained 49% cellulose, 28.3% hemicellulose, 6% extractives and 13.7% AIL on a dry weight basis with 65% moisture content at time of use.

Both sweet and forage sorghums used in this work fall in the genus *Sorghum bicolor* ssp. b*icolor* (Whitfield et al., 2012) with Sugar T sorghum classified as a sweet variety and AF7401 classified as a forage sorghum. Traditionally sweet sorghum varieties were grown for their high sugar content in molasses production operations because of the high sucrose content found in the plants juice (Billa et al., 1997). This higher sugar concentration is reflected in the difference observed between the level of extractives in the sorghum, with at least 10% more extractives (e.g. free sugar) observed in the Sugar T of the AF7401.

Sweet sorghum varieties are similar to forage sorghum varieties but are usually taller, have larger, fleshier stalks and higher in sugar and water content. Typically forage sorghums are grown for use as animal feed because of their ability to produce high biomass yields (Juerg et al., 2009) with high vegetative growth. More specifically, AF7401 is a brown midrib (BMR) genotype sorghum meaning it typically contains less lignin than non-BMR genotypes (Oliver et al., 2005). Lower lignin content as a general characteristic of BMR-6 varities of sorghum would potentially supprt the need for less energy inputs through pretreatment methods prior to hydrolysis to fermentable sugars and fewer phenolic compounds typically resulting from harsh acid based pretreatments that can reduce enzymatic activity of subsequent hydrolysis processes (Heredia et al., 1990; Palmqvist and Hahn-Hagerdal, 2000). This variety compared to the Sugar T also showed higher cellulose content on a dry weight basis.

2.3.2 Sugars Generated from Enzymatic Hydrolysis of Dried Forage Chopped Sugar T Sorghum

The main effects of CTec2, HTec2, Treatment, and time were significant for glucose (p-value < 0.05). The interactions of CTec2*HTec2*Treatment and CTec2*Time*Treatment were also significant for glucose (p-value < 0.05), suggesting that the enzyme combinations used for hydrolysis were important to sugar released for the different sorghum preparations (autoclaved, SA, LN, and LNSA) and the effectiveness of CTec2 was influenced by time. Sterilizing the dried sorghum provided the best case scenario for glucose concentrations in the hydrolysate for each enzyme combination (Figure 2.1 A, B, C) and resulted in significantly greater (p-value<0.05) sugar concentrations in the hydrolysate than non-sterilized preparations (LN, LNSA, SA). Glucose concentrations in the hydrolysate of sterilized samples ranged from 0.88 mmol glucose/g dry sorghum (158.4 mg glucose/g dry sorghum) to 1.24 mmol glucose/g dry sorghum (223.2 mg glucose/g dry sorghum). All preparations at each enzyme loading show greater glucose concentrations in the hydrolysate than the controls (no enzymes) indicating that the presence of the enzymes did effect the release of sugars from the dried sorghum (in addition to those sugars inherent to the enzyme source).

Glucose concentrations in the hydrolysate at time 0 with sterilization (autoclaving) were up to 6.5 times higher than in SA, LN and LNSA preparations because autoclaving was a type of mild pretreatment (Cao et al., 2012) (high temperature, high pressure) that may have contributed to the release of soluble sugars in the sorghum (Figure 2.1 D, E, F) and was the primary reason the treatment interaction with the enzyme factors was significant.

Glucose released within the first 24 hours, on average, resulting in increased glucose concentrations in the hydrolysate that were statistically significant (p-value<0.05) for all preparations within each CTec2 level except for LNSA treatments at CTec2 10 FPU/g. Beyond 24 hours, the amount of glucose released did not increase significantly for all treatments (p-value >0.05) and in most preparations decreased with time. Changes in the glucose concentrations in the hydrolysate, on average, were less evident when sterilized material was used. There were several observations in LN preparations where glucose concentration in the hydrolysate decreased after 48 hours and then increased when measured at 72 hours. This may be related to the destructive sampling method used for the experimental units in combination with timing of hydrolysis in this nonsterile sorghum system. Increased formation of byproducts by contaminant microorganisms from 24 to 48 hours was likely the cause of noticeable decreases in the glucose concentration of the hydrolysate. However, after 48 hours there may have been environmental and nutrient limitations that caused the byproduct formation rate to decline, which, combined with the rate at which glucose was released from the substrate by the enzymes resulted in higher glucose concentrations in the hydrolysate at 72 hours. This hypothesis is supported by the increased formation of acid and solvent byproducts after 48 hours in the LN preparations and the minimal sugar losses in the baseline sterilized preparations where contaminant microorganisms were likely controlled (Figure 2.4).

The presence of more CTec2 was significant to the amount of glucose released regardless of preparation (p-value < 0.05). Within each CTec2 level, HTec2 did not have a significant effect on the amount of glucose released (p-value>0.05). As seen in Table 2-4,

significantly more glucose was released (difference between measured value and initial value) at CTec2 25 FPU/g sorghum than 10 FPU/g sorghum for all treatments (A, LN, LNSA, SA) and at each time step. As expected, the sterilized (autoclaved) preparations resulted in higher total amounts of glucose released on average (including sugars present at time 0) at each time step relative to the additive (LN, SA, LNSA) preparations (p-value < 0.05). Within the sterilized (autoclaved) preparations, there was no statistical difference (p-value>0.05) between the amount of glucose released at CTec2 25 FPU/g and 50 FPU/g after 24 hours suggesting less enzyme and time would be needed for hydrolysis (Figure 2.1; Table 2-4). Hydrolysis carried out with SA and LN additives resulted in statistically similar (p-value>0.05) quantities of glucose being released as a result of enzymatic activity relative to sterilized preparations after 24 hours (Table 2-4). Despite similar potential of SA and LN additives to sterilization as a material preparation, byproduct formation and possible inhibition effects of the additives toward useful microorganisms used during subsequent fermentation operations make these treatments, especially SA, less desirable for sugar production from whole stalk sorghums for conversion to bio-based products.

Table 2-4: Glucose Production from dried sorghum for each material treatment at each CTec2 level over time and across HTec2 levels

Time	Treatment	CTec2 10 mg/g sorghum	CTec2 25 mg/g sorghum	CTec2 50 mg/g sorghum
24	A	98.6^{Aa}	121.1^{ABa}	149.7^{Ba}
	LN	59.9^{Aa}	88.6^{Ba}	112.6^{Ca}
	LNSA	12.6^{Aa}	44.5^{Aa}	46.2^{Aa}
	SA	53.7^{Aa}	80.9^{Ba}	112.0^{Ca}
48	A	91.2^{Aa}	123.9^{ABa}	152.8^{Ba}
	LN	18.7^{Ab}	3.4^{Ab}	9.2^{Ab}
	LNSA	32.6^{Ab}	47.9^{Aa}	49.5^{Aa}
	SA	23.2^{Ab}	57.2^{Bb}	68.5^{Bb}
72	A	69.9^{Aa}	113.5^{Ba}	123.9^{Ba}
	LN	39.4^{Ac}	58.0^{ABc}	77.5^{Bc}
	LNSA	-	-	-
	SA	0.0^{Ac}	43.7^{Bc}	70.6^{Cb}

Note: All values are reported in mg glucose/g sorghum and have been corrected for time 0 concentrations. Different capital letters show significantly (p-value <0.05) different values within material preparation (A, LN, LNSA, SA) and lower case letters show significantly (p-value<0.05) different values within CTec2 loading (10 FPU/g, 25 FPU/g, 50 FPU/g)

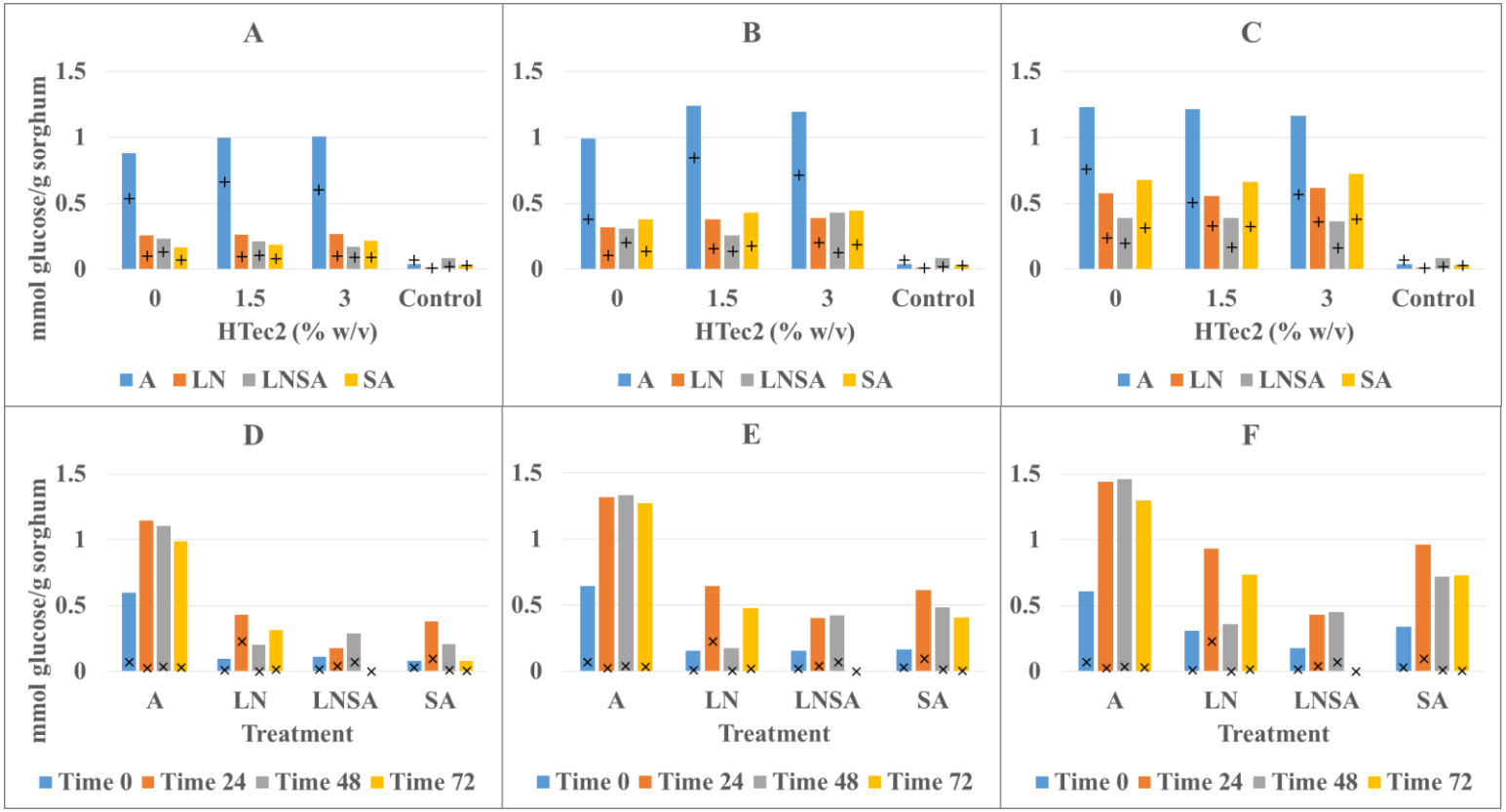

Figure 2.1: Glucose concentrations for different levels of HTec2 and the four material preparations at each CTec2 loading (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g and (C) CTec2 50 FPU/g across time; and for the four different preparation at each CTec2 loading over time (D) CTec2 10 FPU/g (E) CTec2 25 FPU/g (F) CTec2 50 FPU/g; "+" symbols represent Time 0 glucose concentrations; "x" symbols represent no enzyme control glucose concentration

The main effects of CTec2, HTec2, treatment, and time were significant for xylose (p-value < 0.05). The interactions of CTec2*HTec2*Treatment and CTec2*Time*Treatment were also significant for xylose (p-value < 0.05), however with close examination of the lsmeans, the effect of autoclaving as a mild pretreatment and the high initial xylose concentrations in the hydrolysate was great enough to produce these statistically significant treatment interactions. Table 2-5 shows the initial xylose concentrations in the hydrolysate for each material preparation and CTec2 loading level across HTec2 loadings because the additional hydrolysis time and addition of HTec2 did not result in measureable differences in the amount of xylose released for each preparation with the exception of autoclaved material. Within the sterilized preparations, xylose released ranged from 30 and 99 mg glucose/g sorghum for the different CTec2 levels at time 0. Interestingly, the amount of xylose released in SA preparations was statistically similar (p-value<0.05) to sterilized preparations within CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum loading levels. However, losses in hydrolyzed xylose were greater in SA preparations than for sterilized preparations suggesting that SA was not fully effective at inhibiting formation of byproducts, with 8 to 48 mg xylose/g sorghum being lost during the 72 hour hydrolysis period. Of all treatments, only sterilized preparations at CTec2 25 FPU/g sorghum resulted in higher xylose concentrations in the hydrolysate after hydrolysis was complete, with 11.7, 9.2 and 8.4 mg xylose/g sorghum being released after 24, 48 and 72, respectively (data not shown). No statistically significant (p-value>0.05) amounts of xylose were released for LN or LNSA preparations during the 72 hour hydrolysis period.

Table 2-5: Xylose Concentration for Each Material Treatment of Dried Sorghum for each CTec2 level at Time 0 and Across HTec2 levels

Treatment	CTec2 10 mg/g sorghum	CTec2 25 mg/g sorghum	CTec2 50 mg/g sorghum
A	30.4^{A}	36.0^{A}	99.6^{B}
LN	0.0^{C}	0.0^{C}	0.0^{C}
LNSA	0.2^{C}	0.4^{C}	4.5^{C}
SA	8.4^{C}	24.7^{A}	47.6^{B}

Note: All xylose concentrations are in mg xylose/g sorghum

The main effects CTec2, HTec2, Treatment and time (p-value<0.05) were significant for secondary sugars cellobiose and arabinose. The full interaction of CTec2*HTec2*Treatment*Time (p-value<0.05) was also significant suggesting that the enzyme combinations and material preparation (autoclaved, LN, LNSA, SA) were important for the amount of cellobiose and arabinose hydrolyzed during enzymatic hydrolysis (Figure 2.2, Figure 2.3). For sterilized preparations, increased CTec2 loading improved the overall hydrolysis of the dried sorghum, where cellobiose was hydrolyzed over time with statistically higher amounts of cellobiose being released at the CTec2 25 FPU/g sorghum level than the CTec2 10 FPU/ g sorghum and CTec2 50 FPU/g sorghum (p-value < 0.05). Increases in the amount of cellobiose and arabinose hydrolyzed within 24 to 48 hours for the different enzyme combinations along with slight decreases after 72 hours were observed. These observations are an indication that the cellulase was effective in hydrolyzing the cellulose found in sorghum, where with increased activity and time the cellobiose concentrations accumulated in the hydrolysate and upon further and/or more effective hydrolysis that cellobiose was converted to glucose. This accumulation and disappearance of product intermediates is also supported by the glucose concentrations observed in the hydrolysate for

the dried autoclaved material over time (Figure 2.1, Table 2-4). The HTec2 loadings had an effect on the breakdown of hemicellulose in the material based on the arabinose release rate, however, the amount of arabinose released was not statistically different between HTec2 loading levels within the different CTec2 levels for sterilized preparations (Figure 2.2).

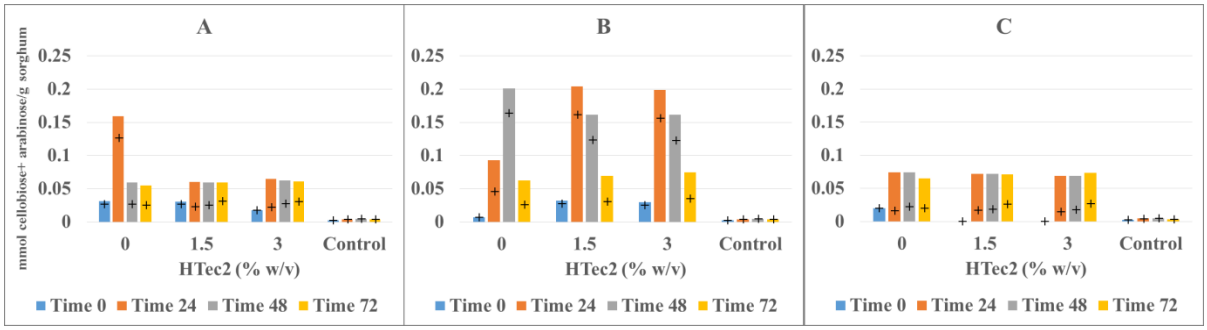

Figure 2.2: Cellobiose and arabinose concentrations for different levels of HTec2 at each time for each preparation and CTec2 loading: (A) Autoclaved CTec2 10 FPU/g (B) Autoclaved CTec2 25 FPU/g (C) Autoclaved 50 FPU/g (+) symbols represent cellobiose concentration for each preparation

LN and SA preparations were statistically similar (p-value>0.05) within each CTec2 loading with higher cellobiose and arabinose concentrations observed in the hydrolysate at CTec2 25 and 50 FPU/g sorghum (Figure 2.3 A, B, C and G, H, I). Within each CTec2 loading level for LN and SA treatments, HTec2 loading did not have an effect on maximum amount of arabinose and cellobiose released during hydrolysis, however, it did have an effect on the rate at which they were released. LNSA preparations, on average, resulted in little to no cellobiose or arabinose release regardless of enzyme loading or time except for at CTec2 25 FPU/g sorghum and HTec2 3% w/v after 24 hours (Figure 2.3 D, E, F). It is not clear why the combination of the LN and SA preparations in LNSA, limit the hydrolysis of the dried sorghum to monosaccharides.

The main effects of time and treatment were significant for the amount of byproducts (lactate, acetate and ethanol) formed ($p<0.05$). The Time*Treatment interaction ($p<0.05$) was also significant suggesting that material preparation had the largest impact on the byproduct formation rate because of the effect the additives had on activity of native microbial contaminants. Increases in the amount of byproduct formed, largely lactate, after 48 hours in SA preparations was likely a result of multiple actions occurring simultaneously during hydrolysis. Charteris et al. (1998) showed that lactic acid bacteria (LAB) are resistant to sodium azide at the 0.03% level which is higher than the sodium azide concentration used in this study. It is likely that at this concentration the sodium azide was eliminating non-LAB gram-negative bacteria and fungi early in the hydrolysis process. Considering LAB are anaerobic (Teuber, 1993) and the sodium azide treatment reduced microbial competition of native flora in the non-sterile treatments, once oxygen became limiting, the LAB were able to consume free sugars, producing acids and resulting in the large increase in byproduct concentrations in the hydrolysate after 48 hours. This also mirrors losses in glucose and xylose concentrations in the hydrolysate during hydrolysis (Figure 2.1).

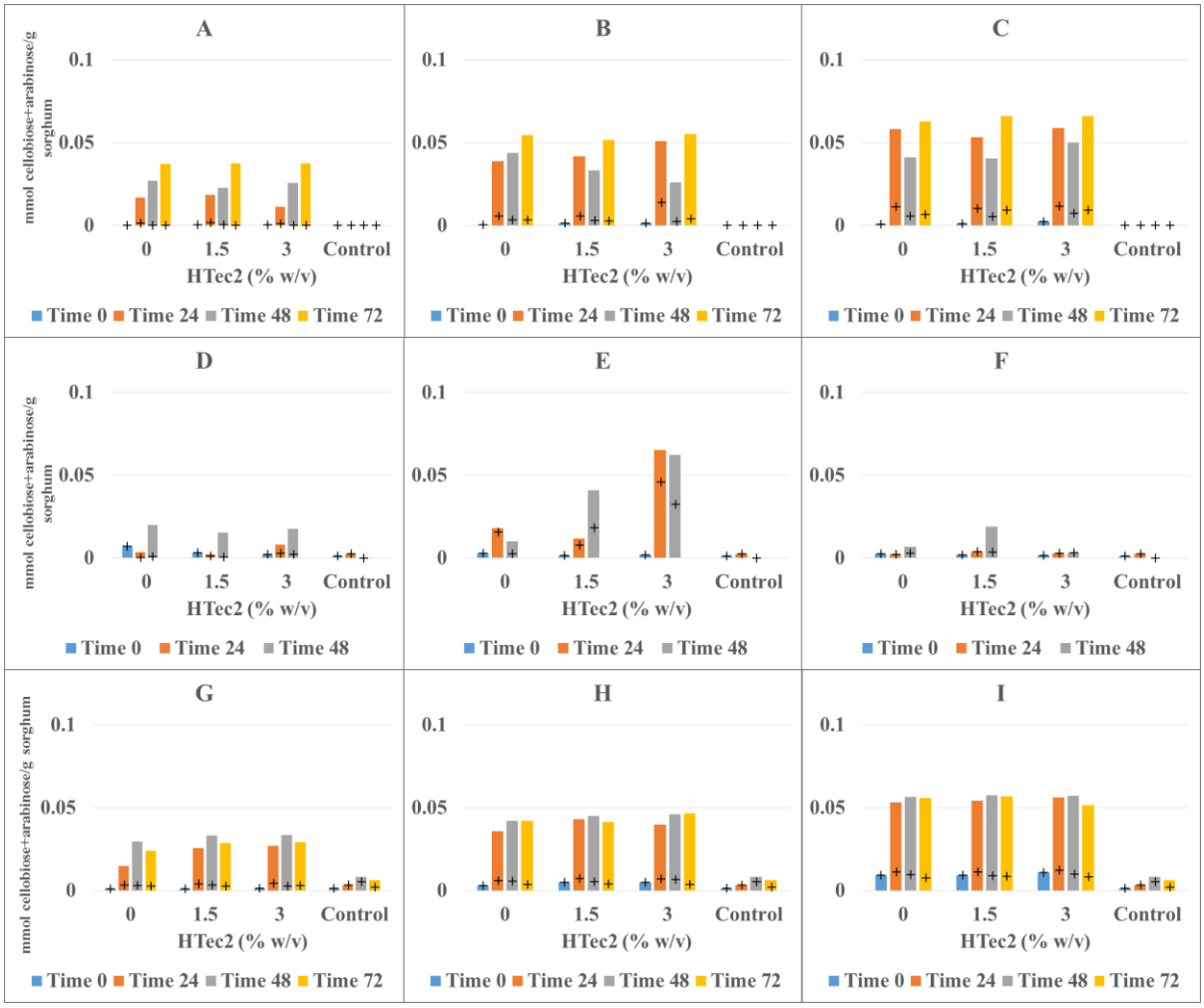

Figure 2.3: Cellobiose and arabinose concentrations for different levels of HTec2 at each time for each preparation and CTec2 loading: (A) LN CTec2 10 FPU/g (B) LN CTec2 25 FPU/g (C) LN CTec2 50 FPU/g (D) LNSA 10 FPU/g (E) LNSA CTec2 25 FPU/g (F) LNSA CTec2 50 FPU/g (G) SA CTec2 10 FPU/g (H) SA CTec2 25 FPU/g (I) SA CTec2 50 FPU/g ((+) symbols represent cellobiose concentration for each preparation

Similar to SA preparations, although statistically lower in byproduct concentration in the hydrolysate (p-value<0.05), lactate was the major byproduct formed for LN preparations despite the fact that lysozyme has been shown to be effective at preventing LAB growth with 8-log reductions per ml being reported after 2 hours (Chung and Hancock, 2000). Lysozyme is an enzyme that is able to lyse bacterial cell walls by hydrolyzing the 1,4-beta linkages

between B-acetylmuranic acid and N-acetyl-D-glucosamine in peptidoglycan and between N-acetyl-D-glucosamine in chitodextrins (Peters et al., 1989). Because gram-positive bacteria have much larger quantities of peptidoglycan, lysozyme is most commonly associated with targeting gram-positive bacteria. Nisin is an antibiotic that causes a rapid outflow of amino acids and Rb+ from the cytoplasm of gram-positive bacteria resulting in decreased membrane potential (Ruhr and Sahl, 1985). Although both lysosyzme and nisin have been reported to target gram-positive bacteria, such as LAB, the observed limited effectiveness of lysozyme-nisin at preventing gram-positive sugar consumption in this study may be related to the concentration used with a solid substrate as well as the hydrolysis pH and temperature (pH 5.0, 50°C) being outside the previously reported conditionsused in other studies, where Chung and Hancock reported use at a pH of 7.2 and temperature of 22°C. However, there appears to be some synergy between lysozyme-nisin and sodium azide at preventing byproduct formation, as seen in Figure 2.4 because LNSA resulted in significantly lower (p-value<0.05) byproduct concentrations than both LN and SA treatments individually at all times. Sodium azide inhibits cytochrome oxidase reactions and can limit cellular respiration in eukaryotes (Lichstein and Soule, 1943) which when used in combination with lysozyme and nisin synergistically addressed challenges with sugar losses by both gram-positive bacteria and eukaryotes (e.g. yeasts).

Although only small quantities of byproducts were formed for sterilized material preparations (Figure 2.4), the majority of the byproducts formed were acetate, as seen in Table 2-6. Interestingly, LN and LNSA preparations did not show increased acetate or lactate percentages within byproducts formed, consisting of roughly 50% acetate and 50%

lactate. However, after 48 hours LN preparations resulted in largely ethanol as the byproduct with 65% of the total byproducts formed being ethanol. While, LNSA preparations after 48 hours showed slightly higher amounts of ethanol as a percentage of the total byproducts but maintained a similar percentage of acetate and a slightly lower percentage of lactate. SA preparations, however, showed that the majority of the total byproducts formed during hydrolysis were lactate most likely because an additive was not present to address the gram-positive bacteria. Data presented in Table 2-6 and Figure 2.4 suggests that LN was effective at preventing lactate formation by LAB during the first 48 hours of hydrolysis but was unable to prevent formation of lactate after 48 hours. LNSA was able to prevent lactate formation during the 48 hour hydrolysis period, although slight increases in ethanol formation were observed. There is opportunity to look at the concentrations used in semi-solid hydrolysis systems to enhance effectiveness of the additives on dried materials.

Table 2-6: Percentage of Total Byproducts Formed Over Time and Adjusted for Initial Byproduct Concentrations in the Hydrolysate for Dried Chopped Sorghum

Treatment	Time	% Acetate	% Lactate	% Ethanol
A	24	76.8	23.5	0
LN	24	35.6	54.9	9.5
LNSA	24	52.7	47.3	0.0
SA	24	38.3	67.2	0
A	48	88.1	12.5	0
LN	48	2.0	32.9	65.1
LNSA	48	49.6	40.5	9.9
SA	48	13.0	84.7	2.3
A	72	95.3	5.0	0
LN	72	5.6	86.7	7.7
LNSA	72	-	-	-
SA	72	12.1	85.9	2.0

It is likely that although shown to be ineffective individually, sodium azide and lysozyme nisin combined eliminate a large enough microbial spectrum to reduce the formation of byproducts microbial growth, or (2) combinations of lysozyme-nisin and sodium azide inhibit enzyme activity on solid substrates resulting in lower soluble sugar concentrations available for byproduct formation (Figure 2.1, Figure 2.2, Table 2-4). Sterilizing the dried sorghum material still resulted in the formation of byproduct, however the amount of byproducts formed appeared to be independent of time and are likely a result of microbial activity occurring prior to hydrolysis. The increases in the amount of sugar that was released in sterilized preparations (Figures 2.1 and 2.2) were also relatively stable over time and were not used to form byproducts as a result of microbial proliferation. Overall, sterilization of the dried sorghum supported greater extent of hydrolysis and sugar yields.

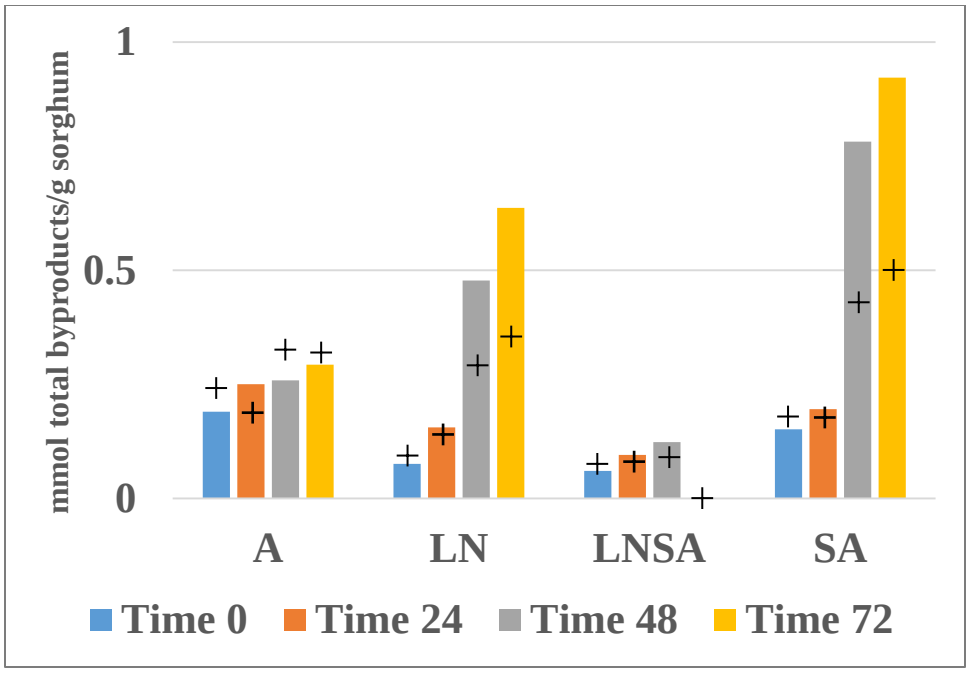

Figure 2.4: Total Byproducts Formed for each material preparation (A, LN, LNSA, SA) at each time (0,24,48,72); (+) symbol represent no enzyme control for each material preparation

2.3.3 Sugars Generated from Enzymatic Hydrolysis of Fresh Forage Chopped AF7401 Sorghum

The main effects of CTec2, Treatment and Time were significant for glucose and xylose (p-value<0.05). The interaction of CTec2*Time*Treatment (p-value<0.05) was also significant for glucose and xylose suggesting that the effectiveness of CTec2 for each material preparation (autoclaved, LNSA, SA) was influenced by time. In addition the CTec2*HTec2 interaction was also significant for xylose suggesting that enzyme combinations had an effect on the amount of xylose released during hydrolysis. Initial glucose concentration in the hydrolysate for sterilized preparations were 1 to 1.6 times higher than all other treatments within each CTec2 group (Figure 2.5). Similar to what was observed in the dried sorghum, the autoclaving process used to sterilize the fresh biomass

mimicked a mild pretreatment altering the structure of the sorghum. The overall initial dry matter in the fresh material was only approximately 0.4 grams and the enzyme loading was based on the total weight. As a result the sugars naturally present in the enzyme stock solutions were relatively high for each experimental treatment unit. This effected the initial sugar concentrations in the hydrolysate, with 95% and 125% percent higher glucose concentrations in the hydrolysate being observed at CTec2 50 FPU/g than at CTec2 25 FPU/g and 10 FPU/g, respectively. For LNSA preparations significant amounts of glucose were released during the first 24 hours for all CTec2 loadings with the glucose concentration in the hydrolysate not changing significantly after 48 hours. Similar to LNSA preparations, SA preparations resulted in glucose being released in the first 24 hours, but experienced decreases in the glucose concentration of the hydrolysate at the 48 hour time period for all CTec2 loading levels. The glucose concentration in the hydrolysate for sterilized preparations did not change after 24 hours for the 25 FPU/g sorghum CTec2 loading, and at CTec2 10 FPU/g sorghum and 50 FPU/g sorghum no increase in the amount of glucose in the hydrolysate was observed until 48 hours.

Higher CTec2 loadings increased glucose hydrolysis yields with the largest increases in glucose concentrations in the hydrolysate being observed at CTec2 50 FPU/g sorghum (Table 2.5) regardless of material preparation. However, lower CTec2 loadings resulted in more active enzymes for sterilized and LNSA preparations, releasing 2.5-4.6 mg glucose/FPU and 4.4-4.8 mg glucose/FPU respectively, suggesting that a higher substrate to enzyme ratio resulted in more available substrate binding sites allowing more efficient use of the enzymes present during hydrolysis. Interestingly, the activity of the cellulase enzymes in

sterilized preparations dropped significantly at CTec2 loading levels greater than 10 FPU/g with 2.5-4.6 mg glucose/FPU being observed at CTec2 10 FPU/g and 0-0.1 mg glucose/FPU at CTec2 50 FPU/g. Enzyme activity for LNSA preparations decreased from 4.4-4.5 mg glucose/FPU to 2.4-2.8 mg glucose/FPU at CTec2 25 FPU/g with no significant change in enzyme activity being observed from the CTec2 25 FPU/g to the CTec2 50 FPU/g loading levels.

Hydrolysis in LNSA preparations resulted in 266.1 mg glucose/g sorghum being released at the CTec2 50 FPU/g sorghum loading level which was statistically greater than the other CTec2 loadings within this preparation. Within the CTec2 50 FPU/g sorghum enzyme loading level, the amount of glucose released was statistically higher than SA and sterilized preparations with the peak concentration of glucose in the hydrolysate being observed after 24 hours. Interestingly, the maximum amount of glucose released for SA preparations were either statistically similar or higher than those measured for autoclaved preparations and occurred within 24 hours of hydrolysis at all CTec2 loadings. For fresh material, sterile preparations appeared to result in slowed glucose release rates. Unlike sterilized and LNSA preparations, SA preparations had significant decreases in hydrolysate glucose concentrations beyond 24 hours, likely a result of proliferation of native microflora. This observation also suggests that lysozyme-nisin has a reasonable effect on inhibiting the activity of the native microorganisms in combination with the preservation effects of sodium azide in fresh sorghum.

The most effective preparation and enzyme loading combination for fresh material was LNSA with CTec2 loading of 50 FPU/g resulting in 65% of the available cellulose being

hydrolyzed into glucose (Table 2-8). Furthermore, within LNSA preparations, increasing CTec2 loading from 25 FPU/g to 50 FPU/g did not result in lower enzymatic activity.

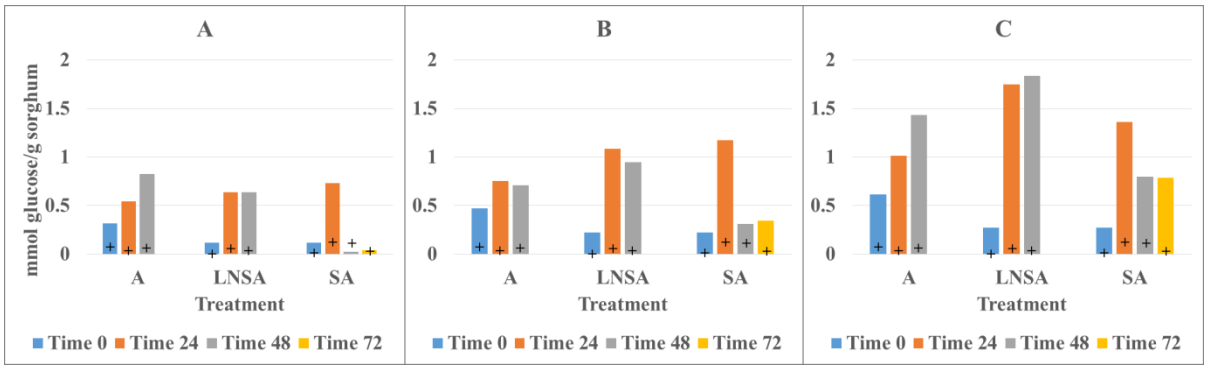

Figure 2.5: Glucose concentration from fresh sorghum for each material treatment (A,LNSA,SA) for each CTec2 loading rate across HTEC2 loading; (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g (C) CTec2 50 FPU/g; (+) symbol represents time 0 glucose concentration

Table 2-7: Glucose Concentration Changes over Time for Fresh Sorghum for each material treatment (A, LNSA, SA) at each time and CTec2 level

Time	Treatment	CTec2 10 FPU/g	CTec2 25 FPU/g	CTec2 50 FPU/g
24	A	41.7	51.4	71.9
	LNSA	94.1	156.0	266.1
	SA	110.6	171.7	196.0
48	A	92.6	43.4	147.5
	LNSA	93.3	131.0	281.9
	SA	-16.5	16.1	94.0
72	A	-	-	-
	LNSA	-	-	-
	SA	-13.8	22.0	92.0

Note: All concentrations are in mg glucose/g sorghum and have been corrected for initial concentrations

Initial xylose concentrations in the hydrolysate for sterilized preparations were statistically similar to those observed for LNSA and SA preparations within the CTec2 10 FPU/g sorghum and 25 FPU/g sorghum loading levels, while it was statistically higher at the CTec2 50 FPU/g sorghum level (Figure 2.6). The amount of xylose in the hydrolysate decreased from its maximum (time 0) for LNSA and SA preparations, on average, as hydrolysis progressed, except for SA preparations at CTec2 10 FPU/g. Sterilized preparations resulted in xylose being released during the first 24 hours of hydrolysis for CTec2 10 FPU/g sorghum and 25 FPU/g sorghum loading levels while the xylose concentration in the hydrolysate did not increase significantly until 48 hours for the CTec2 50 FPU/g sorghum level. All preparations resulted in higher xylose concentrations in the hydrolysate when compared to no enzyme controls suggesting that inclusion of CTec2 effected xylose release regardless of material preparation. Increased CTec2 loading resulted

in higher xylose concentrations in the hydrolysate regardless of preparation. There are several observations where the amount of xylose observed in the hydrolysate decreases at one time point and increases at another. This may be related to byproduct formation rate by contaminant microorganisms being higher than the rate at which xylose was being released by CTec2 in SA preparations. Changes in nutrient availability over time may decrease the rate at which the microorganisms are consuming xylose to form byproducts resulting in xylose concentrations increasing due to continued enzymatic hydrolysis. This result is similar to what was observed in SA preparations on dried sorghum.

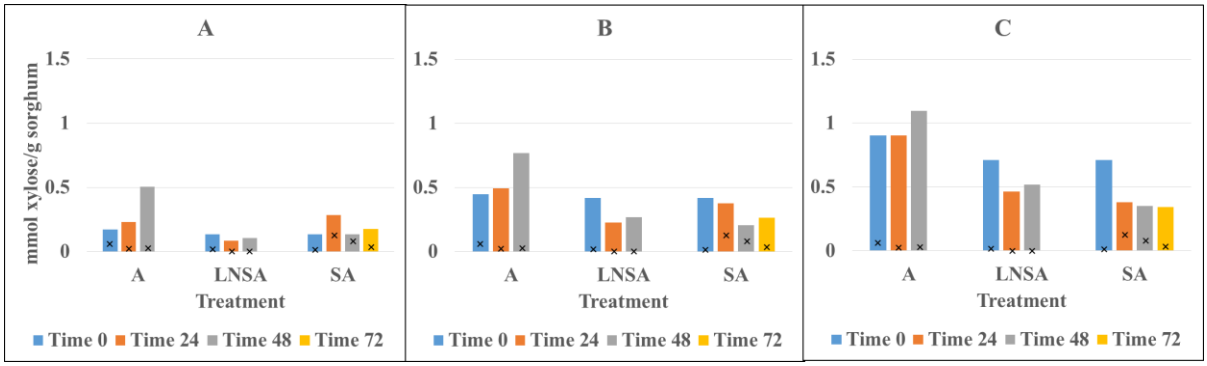

Figure 2.6: Xylose concentration for fresh sorghum for each material preparation (A, LNSA, SA) at each CTec2 loading across HTEC2: (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g (C) CTec2 50 FPU/g: (x) symbol represents no enzyme control for each material treatment

Although significant (p-value<0.05) on the amount of xylose released, the interaction of CTec2*HTec2 appears to be more related to the CTec2 loading levels than the HTec2 loading levels (Figure 2.7), with significant increases in the xylose concentration in the hydrolsate occurring with each increase in CTec2 loading. Small differences in the average amount of xylose in the hydrolysate for different HTec2 loading rates are seen in the data but

they are not significantly different (p-value>0.05) within each CTec2 loading rates. This is supported by the fact that HTec2 is not significant (p-value>0.05) as a main effect on xylose released.

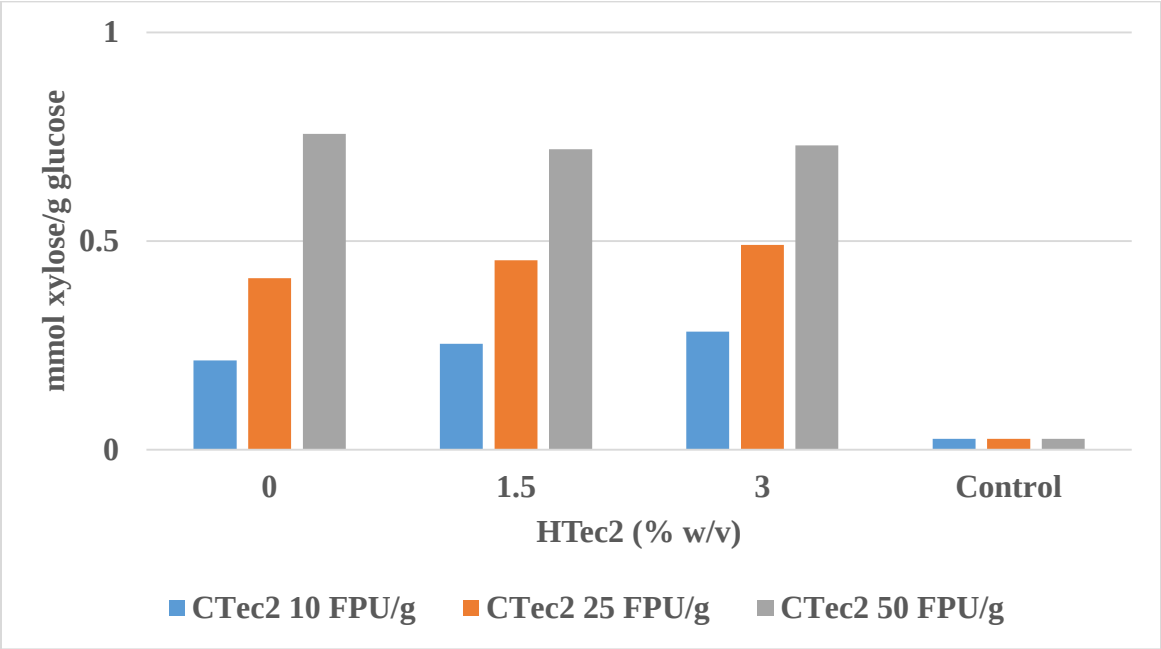

Figure 2.7: Xylose concentration for fresh sorghum for each HTec2 loading at each CTec2 level across Treatment and Time

The main effects of CTec2, Treatment and Time (p-value<0.05) were significant for cellobiose and arabinose concentrations in the hydrolysate. The interaction of CTec2*Time*Treatment (p-value<0.05) was also significant for arabinose suggesting that the effectiveness of CTec2 within material preparations (autoclaved, LNSA, LN) was influenced by time (Figure 2.8). No significant factorial interactions were observed for cellobiose. All preparations resulted in higher arabinose concentrations in the hydrolysate beyond the control over time as CTec2 loading levels increased. Arabinose concentrations in the

hydrolysate were higher for sterilized preparations than for LNSA and SA preparations at increased CTec2 loading levels (50 FPU/g) after 72 hours. LNSA preparations did not result in significant amounts of arabinose being released until 48 hours regardless of the CTec2 loading and the with peak yields in the hydrolysate being significantly less than those observed for both SA and autoclaved preparations within the same CTec2 loading level. The lower CTec2 loading levels for LNSA and SA preparations showed increased arabinose release rates, while the CTec2 50 FPU/g sorghum levels showed delayed hydrolysis with peak arabinose concentrations in the hydrolysate occurring after 48 hours. SA preparations at CTec2 10 FPU/g sorghum and CTec2 25 FPU/g sorghum showed decreases in arabinose concentrations in the hydrolysate after 24 hours while concentrations in the hydrolysate were stable at CTec2 50 FPU/g sorghum. This may be the result of the higher enzyme loading rate releasing arabinose more rapidly than byproducts were being produced by microbial contaminants as indicated by decreases in the amount of other sugars in the hydrolysate in the SA treatments on fresh sorghum.

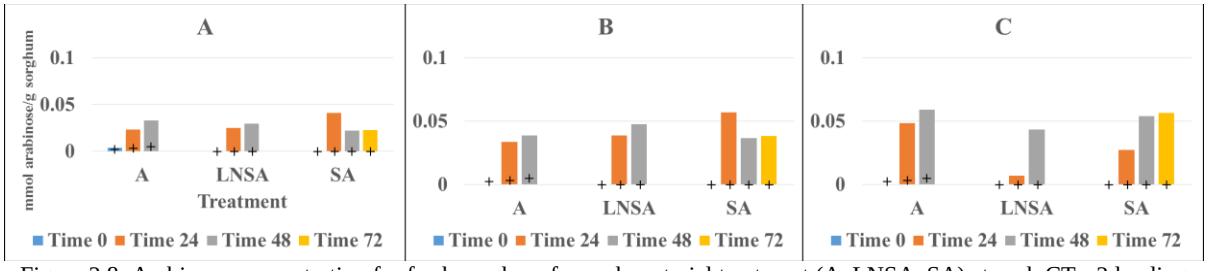

Figure 2.8: Arabinose concentration for fresh sorghum for each material treatment (A, LNSA, SA) at each CTec2 loading level across HTEC2 loading: (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g (C) CTec2 50 FPU/g

The main effects of Time and Treatment were significant (p-value<0.05) for total byproducts (acetate, lactate and ethanol). The CTec2*HTec2*Treatment*Time interaction

was also significant (p-value<0.05) suggesting that enzyme combinations effected the rate at which byproducts were formed differently for each material preparation (autoclaved, SA, LNSA). LNSA preparations resulted in similar byproduct concentrations in the hydrolysate to sterilized preparations, and were less than SA preparations, indicating that LNSA was as effective as autoclaving in inhibiting naturally occurring microflora in fresh forage chopped sorghums (Figure 2.9).

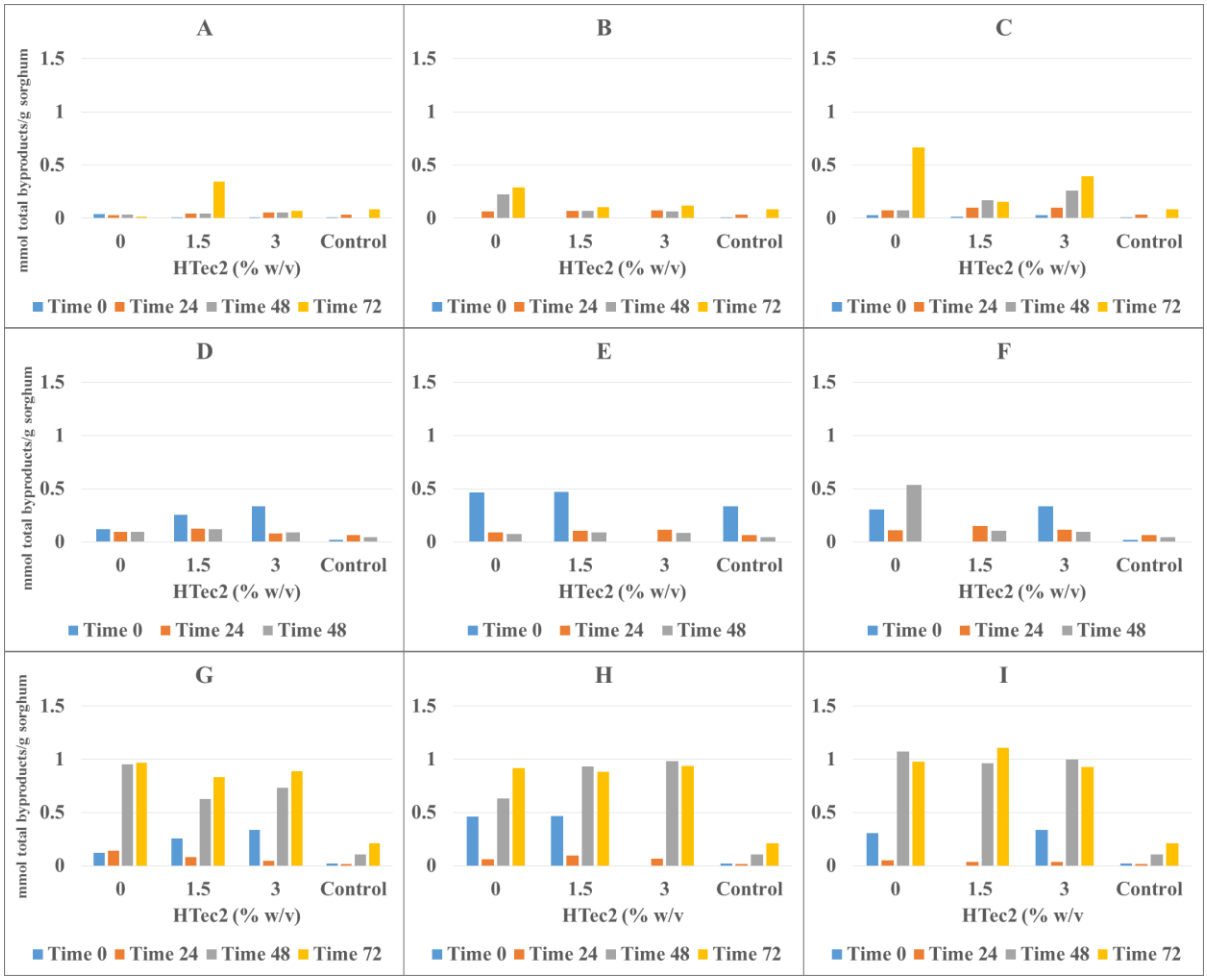

Figure 2.9: Total Byproduct concentrations for different levels of HTec2 at each time for each preparation and CTec2 loading: (A) Autoclaved CTec2 10 FPU/g (B) Autoclaved CTec2 25 FPU/g (C) Autoclaved 50 FPU/g (D) LNSA CTec2 10 FPU/g (E) LNSA CTec2 25 FPU/g (F) LNSA CTec2 50 FPU/g (G) SA CTec2 10 FPU/g (H) CTec2 25 FPU/g (I) CTec2 50 FPU/g

Slightly higher initial byproduct concentrations in the hydrolysate for LNSA preparations (Figure 2.8 D, E, F) was likely the result of spoilage during the lapse in processing the biomass after harvest as time 0 samples were prepared and destructively sampled after all other LNSA preparations had taken place (~8 hours post-harvest). This is an example of the sensitivity of sorghums to soluble sugar losses by microbial action. As a result glucose yields and enzyme efficiency numbers calculated were adjusted using time 0 data values from SA preparations (Table 2-8 and Table 2-9). Increases in the amount of byproducts formed from 24 to 48 hours were very large in sodium azide preparations (Figure 2.9 G, H, I) suggesting that the 0.02% w/v concentration was not effective at preventing contamination and sugar utilization by proliferating microflora during byproduct formation, particularly gram-positive bacteria. Similar to dried material, LNSA preparations were able to effectively prevent proliferation of gram-positive bacteria (e.g. LAB, clostridia, bacilli) and eukaryotes (e.g. yeasts) with minimal byproduct formation occurring during hydrolysis. Increased concentrations of byproducts in the hydrolysate at higher CTec2 loading was observed indicating that higher sugar concentrations in the hydrolysate generated from effective hydrolysis allowed more rapid growth of contaminants. This result was observed across all material preparations of fresh chopped sorghum. Use of LNSA to prevent microbial spoilage of fresh sorghums was observed to be relatively effective and did not appear to be the limiting factor in the amount of sugar released during enzymatic hydrolysis of fresh and dried sorghum.

2.3.4 Enzyme Efficiency and Net Cellulose to Glucose Conversion

Differences in enzyme efficiency between the different preparations for dried sorghum were observed (Table 2-8). For both fresh and dried sorghums, enzyme efficiency appeared to be highest during the first 24 hours of hydrolysis (Table 2-9) for non-LNSA preparations. While LNSA was able to minimize byproduct formation by naturally occurring microorganisms, it seemed to create a delay in enzyme activity towards dried sorghum and in some cases fresh sorghum (CTec2 50 FPU/g sorghum). In addition, a significantly lower enzyme efficiency (p-value <0.05) was observed between preparations and with increases in CTec2 loading level for the dried sorghum. However, for fresh sorghum LNSA preparations showed the difference in enzyme efficiencies among the preparations was inconsistent. Apparent inhibition of enzyme activity at the lower CTec2 loading by LNSA appears to be overcome at CTec2 25 FPU/g sorghum loading and above, although the reason for this phenomena is still unclear.

For sterilized preparations of dried sorghum significantly higher (p-value<0.05) enzyme efficiencies were observed than in non-sterilized preparations within all CTec2 loading levels. LN preparations had significantly higher (p-value<0.05) enzyme efficiency at CTec2 10 FPU/g sorghum than LNSA preparations but were similar at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum. Interestingly, sterilization of fresh material resulted in lower enzyme efficiency (p-value<0.05) than both LNSA and SA preparations at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum but were statistical similar (p-value>0.05) to LNSA and SA preparations at CTec2 10 FPU/g sorghum. Considering both sorghum materials, the trends in enzyme efficiency as it relates to net glucose release with the

presence of additives were inconclusive. Changes in the physical structure during sterilization (autoclaving), similar to mild pretreatment (Cao, et al., 2012), likely resulted in more easily accessible cellulose than non-sterilized preparations. As a result, lower enzyme loadings were capable of efficiently hydrolyzing the cellulose to glucose and higher enzyme loadings were unnecessary (e.g. lower efficiency of the enzymes, Table 2-8). This observation is similar for the additive preparations and is consistent with findings from Sattler et al. (1988) who showed that enzyme loadings beyond 10 FPU/g dry substrate resulted in insignificant increases in glucose release during the first 24 hours of hydrolysis.

Table 2-8: Amount of glucose released on an enzyme activity basis (mg glucose/FPU) for CTec2 10 FPU/g, CTec2 25 FPU/g and CTec2 50 FPU/g for each preparation (A, LN, LNSA, SA)

CTec2 Loading Level	**Treatment**	**mg glucose/FPU**		**Maximum Glucose Released in mg per FPU (Time)**	
		Dried	**Fresh**	**Dried**	**Fresh**
10	A	8.2-11.6	2.5-4.6	11.6 (24)^{Aa}	4.6 (24)^{Aa}
	LN	2.0-7.0	-	7.0 (24)^{Ba}	-
	LNSA	1.5-3.8	4.4-4.5	3.8 (48)^{Ca}	4.5 (24)^{Aa}
	SA	0.0-6.3	0-4.8	6.3 (24)^{BCa}	4.8 (24)^{Aa}
25	A	5.3-5.7	0.6-1.4	5.7 (24)^{Ab}	1.4 (24)^{Ab}
	LN	0.2-4.6	-	4.6 (24)^{Bb}	-
	LNSA	2.1-2.3	2.4-2.8	2.3^{Ba} (48)	2.8 (24)^{Bb}
	SA	2.0-3.8	0.3-3.0	3.8 (24)^{Bb}	3.0 (24)^{Bb}
50	A	2.9-3.6	0-0.1	3.6 (24)^{Ac}	0.1 (24)^{Ab}
	LN	0.2-2.6	-	2.6 (24)^{BCb}	-
	LNSA	1.1-1.2	2.8-2.9	1.2 (48)^{Bb}	2.9 (48)^{Bb}
	SA	1.6-2.6	0.8-1.7	2.6 (24)^{Ca}	1.7 (24)^{Cc}

Note: Different capital letters represent significantly different values within CTec2 loading level (10 FPU/g, 25 FPU/g, 50 FPU/g) and lowercase letters represent significantly different values within each material preparation (A, LN, LNSA, SA). Statistical comparisons were not made between fresh and dried material because the varieties were different.

Sterilized preparations of dried material resulted in the highest net cellulose to glucose conversion with up to 40% conversion being achieved (Table 2-9). Additive preparations for dried sorghum resulted in approximately 10% less net cellulose to glucose conversion. These lower conversion efficiencies may be a result of the lower enzyme efficiency/activity in LNSA preparations. For LN and SA preparations, the lower enzyme efficiencies in combination with poor effectiveness of the additives in limiting microbial growth of natural microflora contributed to soluble sugar losses and reduced net conversion efficiency numbers. Overall increases in enzyme loading for dried sorghum material resulted in improved conversion, with maximum values occurring in the first 24 hours. Enzyme-substrate-microbe interaction challenges were more profound in the later time steps affecting conversion values and available sugars in the hydrolysate.

Maximum net cellulose to glucose conversion for fresh material was variable for the different preparations with the highest net conversion (67%) occurring after 48 hours for LNSA preparation at CTec2 50FPU/g (Table 2-9). For additive preparations the conversion efficiency improved with higher CTec2 loading, while the additional enzyme activity did not improve conversion of cellulose to glucose within the sterilized preparations, suggesting that the benefits of sterilization of fresh material to alter substrate structure and potential eliminate the natural microflora prior to hydrolysis did not improve net cellulose to glucose conversion. Use of LNSA as an additive to prevent microbial proliferation during hydrolysis of fresh sorghum appears to be a better alternative than sterilization.

Considering this is a net conversion value, the higher byproduct formation in sterilized preparations contributes to the lower glucose release number and decreases the observed

conversion efficiency. The complexity of the system to account for sugars losses to byproducts in the hydrolysate for a single sugar (e.g. sucrose, fructose, glucose, xylose, cellobiose) makes determination of an absolute percent conversion number difficult. Since glucose is the primary sugar of interest, the net value at least gives an idea of what can be effectively achieved. Although net cellulose to glucose conversion in some cases was as high as 67% in fresh material with losses in glucose due to microbial proliferation, not all of the available cellulose was hydrolyzed to glucose during hydrolysis. Furthermore, the data suggested that increasing enzyme loading would not increase the cellulose to glucose conversion substantially. Investigations into mild pretreatment of fresh material in combination with use of additives discussed in this study may help to further hydrolyze cellulose. For dried material the maximum cellulose to glucose conversion was 40% suggesting that there was significantly more cellulose available for conversion. As was the case with fresh, investigations into combination of additives and mild pretreatment should be investigated to try and increase the cellulose to glucose conversion efficiency.

Table 2-9: Net Cellulose to Glucose Conversion Efficiency by Enzymatic Hydrolysis of Dried Sugar T and Fresh AF7401 Sorghums

Treatment	CTec2 (FPU/g w.w.)	Maximum Cellulose to Glucose Conversion (%)	
		Dried	Fresh
A	10	25.9 (24)	22.9 (72)
	25	32.6 (48)	15.2 (72)
	50	40.2 (48)	14.5 (48)
LN	10	15.7 (24)	N/A
	25	23.3 (24)	
	50	29.6 (24)	
LNSA	10	8.6 (48)	20.8 (24)
	25	12.6 (48)	32.6 (24)
	50	13.0 (48)	67.4 (48)
SA	10	14.1 (24)	22.5 (24)
	25	21.3 (24)	35.0 (24)
	50	29.4 (24)	40.0 (24)

2.4 Conclusion

If possible, fresh AF7401 sorghum would be ideally suited for use in hydrolysis systems but immediate use after harvest is imperative but heating of the material may create challenges with the enzymes ability to hydrolyze the sorghum as observed in the sterilized preparations. However, pretreatment of fresh sorghum may still show an added benefit. Addition of LNSA to address the naturally occurring microflora from affecting the fresh material may show advantages to overall conversion. Increasing CTec2 resulted in higher cellulose to glucose conversion but the cost benefit analysis of using high enzyme loadings should be fully explored.

Overall net conversion of dried Sugar T sorghum was incomplete with the enzyme and time treatment combinations used in this study. Drying of sorghum may impact the overall structure such that a pretreatment would be needed to enhance overall conversion. The combined additives of lysozyme, nisin and sodium azide seemed to minimize byproduct formation from the sugars that were present, however, the poor overall conversion would suggest that other factors outside of enzyme loading and time may be necessary. The natural microflora in sorghum poses a problem regardless of storage method. As processes continue to develop, methods to manage these microbial communities will be necessary.

For fresh material, use of HTec2 may not be necessary but it does appear to have some positive benefits to hydrolysis of dried Sugar T sorghum but the cost-benefit ratio needs to be evaluated.

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Chapter 3 Enzymatic Hydrolysis of Ensiled Forage Chopped Sorghum

3.1 Introduction

Sorghums (biomass, sweet, forage types) have the potential to provide enough sugar, starch and lignocellulosic material to adequately supplement lignocellulosic conversion processes for bioenergy and bioproduct production. Parrish et al (1985) and Yosef et al (2009) reported that grains generated from sweet sorghum production produced 1.99-5.17 Mg/Ha on a dry weight basis of biomass with anywhere from 0.64-1.91 Mg/Ha of this being starch 40 days after anthesis. Yosef et al (2009) reported 4-11.5 Mg/Ha of cellulose and hemicellulose for sorghum stalks and leaves with the stems consisting of 48-52% cellulose, 40-50% hemicellulose and 3-9% lignin and the leaves consisting of 40-49% cellulose, 46-53% hemicellulose and 6-10% lignin. In addition to its ideal composition, favorable agronomic characteristics make it increasingly attractive for use as a dedicated bioenergy feedstock. Sorghums have shown the ability to grow to maturity rapidly, with multiple harvests off the same root possible within a single growing season in warmer climates, require low fertilizer and water inputs when compared to other biomass crops, grow well on marginal soils, and produce relatively high amounts of biomass.

Despite its advantages, sorghums have not seen a large role in current ethanol and biorefinery processes in the US, however as the demand for alternative lignocellulosic feedstocks for biobased fuels and products increases sorghum will be a relevant crop. Challenges associated with postharvest storage, handling and seasonal availability of sorghums impact the feasibility of its integration into commercial processing operations.

Making the challenges of seasonal availability and rapid soluble sugar losses even more challenging is the fact that harvesting method can accelerate the spoilage of the crop as well. Veal et al (2011) showed that harvest methods such as forage chopping, which makes use of existing equipment, expose the sugar-rich pith to the environment and result in more rapid decline of sugars than cutting whole stalks at the root and keeping it intact. However, cutting, transporting and processing whole, intact stalks requires either more labor or specified equipment dedicated to harvesting sweet sorghum. As a result of the rapid decrease in sugar content in chopped sorghum (up to 50% of its readily available fermentable sugars within 24 hours after harvest) (Veal et al. 2011) facilities must be able to process large quantities of sorghum in very short periods of time or identify a storage approach that has minimal impact on the conversion process.

One potential way to combat the problems associated with the rapid deterioration of fermentable sugars in sorghums is to use ensiling as a storage approach. As stated by Philipp et al (2007), the goal of ensilage is to prevent deterioration of plant materials through lactic acid fermentation under anaerobic conditions. During the ensiling process, lactic acid forming bacteria (LAB) consume sugars and produce a mixture of acetic acid and lactic acid which increases the hydrogen ion concentration lowering the pH to inhibit microbial activity (Whittenbury et al. 1967). Using ensiling as a long-term storage method could allow smaller biorefineries to use ensiled material and then supplement that with fresh material when it is seasonally available. In addition, reduction of the soluble sugar levels can be beneficial to those processes that are designed to make use of the lignocellulosic fractions and are inhibited by higher sugar concentrations.

Significant sugar losses are common in ensiling processes, however, due to its high soluble sugar content, sorghums are able to undergo the ensiling process without losses to the lignocellulosic fraction of the biomass. In an attempt to lessen these losses, some studies (Cundiff, 1992) have looked at pressing the juice for fermentation then ensiling the bagasse but it is unclear whether there is enough fermentable sugar left in the bagasse to complete the ensiling process. Other attempts to mimic the ensiling process, such as introducing formic acid (Schmidt et al, 1997) to inhibit microbial activity, have shown success but these processes require costly additives that can be problematic when it comes to waste disposal. Although losses in typical ensiling processes result in up to 50% of the free fermentable sugars, the use of ensiling as a long term storage method may have benefits that outweigh those losses, especially for processes targeting lignocellulosic conversion for bio-based products.

In addition to the long term storage benefits of ensiling, another benefit that should be noted is the effect ensiling has on the composition of the lignocellulosic material. Linden et al., (1987) showed that 65% of the original fermentables available in wilted sweet sorghum were preserved during a five month period. Furthermore, the authors of that study went on to show that both pressed and wilted sweet sorghum were more efficiently hydrolyzed during enzymatic hydrolysis following ensiling. Ultimately, it was concluded that the increased efficiency of the enzymatic hydrolysis due to the pretreatment effects ensiling had on the biomass made up for the fermentable sugar losses. However, this study only looked at the effects ensiling had on pressed sweet sorghum bagasse and sweet sorghum that was allowed to wilt in the field. While this study showed promise it must be noted that the fractionation

of the sweet sorghum stalk for the pressed material followed by subsequent grinding added costly steps to the conversion process that may not be feasible for commercial application. Furthermore, the overnight drying to decrease moisture content in the wilting process would allow for sugar losses due spoilage by native microflora.

The overall goal of this work was to examine the feasibility of using forage chopped, whole stalk, ensiled sorghum as an approach to effectively use current machinery, supply of biomass feedstock and reduce costly unit operation for sorghum based conversion processes.

Specific objectives for this study were: (1) Evaluate the impacts of cellulase and hemicellulase loading in terms of final sugar concentration (2) Investigate whether additional additives are needed beyond the ensiling process to inhibit native microflora

3.2 Materials and Methods

3.2.1 Sorghum Feedstock

Sorghum (*Sorghum bicolor*) cultivar AF-7401 was harvested during the 2014 cropping seasons, forage chopped, stored in vacuum sealed bags on ice (0.5 inch) and processed within 24 hours after harvest. Material was placed in sealed ziploc bags, with air removed and wrapped tightly in multiple layers of duct tape to mimic sealed ensilage bags. The ensilage process was allowed to proceed uninterrupted for 5 months prior to use in this study. Sorghum composition analysis was completed using a modified NREL procedure developed by Whitfield et al (2014).

3.2.2 Experimental Design

Enzymatic hydrolysis experiments were completed on ensiled forage chopped sorghum. The effects of cellulase and hemicellulase enzyme loading levels (CTec2 10

FPU/g, CTec2 25 FPU/g, CTec2 50 FPU/g) and hydrolysis time (0, 12, 24, 36, 48,and 72 hours) were investigated for ensiled forage chopped sorghum prepared as sterilized material (autoclaved) and with additives to inhibit naturally occurring microflora, , 1) lysozyme and nisin and 2) sodium azide, lysozyme and nisin combinations. Each treatment combination and controls (no enzyme) were completed in triplicate and were destructively sampled. Response variables were measured over time and included glucose, sucrose, fructose, cellobiose, xylose, arabinose, lactate, acetate and ethanol concentrations. Analysis of variance for main and interaction effects and pairwise comparisons between preparations within were evaluated in SAS. Enzyme (CTec2, HTec2) loading levels were combined to evaluate the effect enzyme loading, material treatment and time had on sugar and byproduct formation versus controls using Proc GLM. To determine differences between treatment groups controls were removed from analysis prior to running Proc GLM and comparing LS means for factorial interactions of CTec2, HTec2, treatment and time.

3.2.3 Enzymes and Chemical Additives

Table 3-1: Enzyme and Chemical Additive Stock Solutions Added to Treatments

Name	Composition	Treatments Used In
Buffer A	10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl	Autoclaved, Lysozyme Nisin
Buffer B	10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl 0.02% w/v Sodium Azide (20 mg/L)	Lysozyme Nisin Sodium Azide
CTec2 Stock	40 ml Buffer A or Buffer B, 4 ml CTec2 (145 FPU/ml)	Buffer A- Autoclaved, Lysozyme Nisin Buffer B- Lysosyme Nisin Sodium Azide
HTec2 Stock	39 ml Buffer A or Buffer B, 1 ml HTec2 (90 IU/ml)	Buffer A- Autoclaved, Lysozyme Nisin Buffer B- Lysosyme Nisin Sodium Azide
Lysozyme Nisin Stock	100 ml Buffer A or Buffer B, 1125 mg/L Lysozyme, 375 mg/L Nisin	Buffer A- Lysozyme Nisin Buffer B- Lysosyme Nisin Sodium Azide

Two different buffers were made for sodium azide and non-sodium azide treatments, respectively to accommodate the addition of multiple additives. Buffer A (10.7 g/l sodium citrate (0.05 M), adjusted to pH 5.0 using HCl) was used for all treatments that did not contain sodium azide and buffer B (10.7 g/l sodium citrate (0.05 M), 0.02% w/v Sodium Azide (20 mg/l) adjusted to pH 5.0 using HCl) was used for all treatments that did contain sodium azide. Enzyme stock solutions were made such that 1 ml of CTec2 enzyme stock would be the equivalent of 10 FPU/g material (40 ml Stock A or Stock B depending on treatment, 4 ml CTec2). The activity of CTec2 was 145 FPU/ml as determined using the filter paper assay developed by Adney and Baker (1996). HTec2 stock solution was made

with 1 ml of HTec2 stock solution and 39 ml Stock A or Stock B depending on treatment. The HTec2 activity was 90 units/ml as determined using a xylanase activity assay. For treatments containing lysozyme and nisin, a stock solution was made such that 1 ml of stock solution would provide 112.5 µg/ml of lysozyme and 37.5 µg/ml of nisin (112.5 mg lysozyme and 37.5 mg of nisin, 100 ml Stock A or Stock B depending on treatment).

Table 3-2: Cellulase activity in the presence of additives, additive combinations and common byproducts

Additive	FPU/ml
Lysozyme	181
Nisin	200
SA	201
Acetate	194
Lactate	138
LN	210
LNSA	192
Control	204

3.2.4 Enzymatic Hydrolysis

Amount of material added to falcon tubes was 1.25g ensiled AF7401 material (80% moisture content, 0.25 g dry sorghum). For sterilized treatments, falcon tubes were autoclaved (121 C, 19 psi) for 60 minutes on a liquid cycle. For non-sterilized treatments, Falcons tubes were UV sterilized for 1 hour in a biosafety cabinet. CTec2 and HTec2 were added as 1,2.5,5 mls (10 FPU/g material, 25 FPU/g material, 50 FPU/g material), and 0,0.5,1 mls (activity of each) to the appropriate falcon tubes, respectively for ensiled AF7401. For treatments that received additives, 13-18 ml by increments of 0.5 ml of buffer B (0.05 M Sodium Citrate buffer x 20 mg/L sodium azide, pH 5) were added to lysozyme nisin sodium azide treatments, 13-18 ml by increments of 0.5 ml of buffer A (0.05M, pH 5) were added to

each falcon tube to make up to 20 ml. Falcon tubes were incubated in a shaking water bath at (50ºC, 50 RPM) and destructively sampled at times (12, 24, 36, 48 and 72 hours). Time zero samples were sampled immediately after the addition of enzymes and additives (without incubation). Samples were processed by centrifugation (2731 x g, 10 min) and hydrolysate aliquots (2 ml) were removed and stored at -80C for HPLC analysis.

3.2.5 HPLC Analysis

The response variables glucose, sucrose, fructose, xylose, cellobiose, arabinose, lactate, acetate and ethanol were determined using high performance liquid chromatography (HPLC). HPLC samples were thawed, centrifuged (14908 x g, 10 min) and filtered through 0.2µ Whatman (Maidstone, UK) syringe filters prior to analysis. A Phenomenex (Torrance, CA) Rezex ROA column (300mm x 7.8 mm) at 55ºC in 50-minute runs with 0.6 mL/min HPLC water (Sigma-Aldrich) containing 5 mM sulfuric acid as the eluent was used to measure glucose, xylose, arabinose, cellobiose, acetic acid and lactic acid concentrations by refractive index detection (RID) using. A Phenomenex RPM column (300mm x 7.8 mm) at 85 ºC using HPLC water (Sigma, Chromasolv) as the eluent (0.6 ml/min) was used to measure glucose, fructose and sucrose concentrations by RID. This procedure was similar to the one used by Whitfield et al (2014).

3.3 Results and Discussion

3.3.1 Sugars Generated from Enzymatic Hydrolysis of Ensiled Forage Chopped AF7401 Sorghum

After ensiling was complete the composition of sorghum cultivar AF7401 was 45% cellulose, 34% hemicellulose and 16% acid insoluble lignin on a dry-weight basis with a

moisture content of 80%. Ensiled AF7401 sorghum had higher hemicellulose, moisture content and lignin compositions than fresh AF7401. Sorghum variety AF7401 is a brown midrib (BMR) genotype sorghum meaning it typically contains less lignin than non-BMR genotypes (Oliver, et al., 2005). Use of BMR-6 varities of sorghum would potentially support the need for less energy inputs through pretreatment methods prior to hydrolysis to fermentable sugars and fewer phenolic compounds typically resulting from harsh acid base pretreatments that can reduce enzymatic activity of subsequent hydrolysis processes. (Heredia, et al., 1990; Palmqvist and Hahn-Hagerdal, 2000).

The main effects of CTec2, HTec2 and Time were significant for the amount of glucose and xylose released (p-value<0.05). The full interaction of CTec2*HTec2*Treatment*Time was also significant (p-value<0.05) suggesting enzyme combinations and material preparation (autoclaved, LN, LNSA) had an effect on soluble sugar release rates for ensiled sorghum (Figure 3.1). Glucose concentrations in the initial hydrolysate from all material preparations were higher overall with an increase in CTec2 loading rate at time 0 as a result of the glucose present in the enzyme formulation itself, however the enzymes were effective in hydrolyzing cellulose as the concentration of glucose in the hydrolysate increased with time. This is also evident in comparison of glucose in the different treatment combinations to the respective controls (Figure 3.1).

Sterilized preparations were completed to serve as a baseline for "best" removal of any potential microbial contaminants resulting after the ensilage process that may contribute to sugar losses during enzymatic hydrolysis. Sterilized material preparations hydrolyzed cellulose to glucose fastest during the first 24 hours with decreases or statistically similar

glucose concentrations in the hydrolysate occurring in the following 48 hours (Figure 3.1 A, B, C). At low CTec2 loading levels (10 FPU/g sorghum) HTec2 appeared to have a more pronounced effect on the amount of glucose released than at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum levels for sterilized preparations, where the effect was not evident. The decreases in glucose concentrations in the hydrolysate for sterilized preparations at higher enzyme loading levels (CTec2 25 and CTec2 50) were not as large as those observed at CTec2 10, suggesting that glucose was being released at a rate similar to the rate at which byproducts were being formed by microbial contaminants (likely cause of sugar losses) at higher CTec2 loading levels.

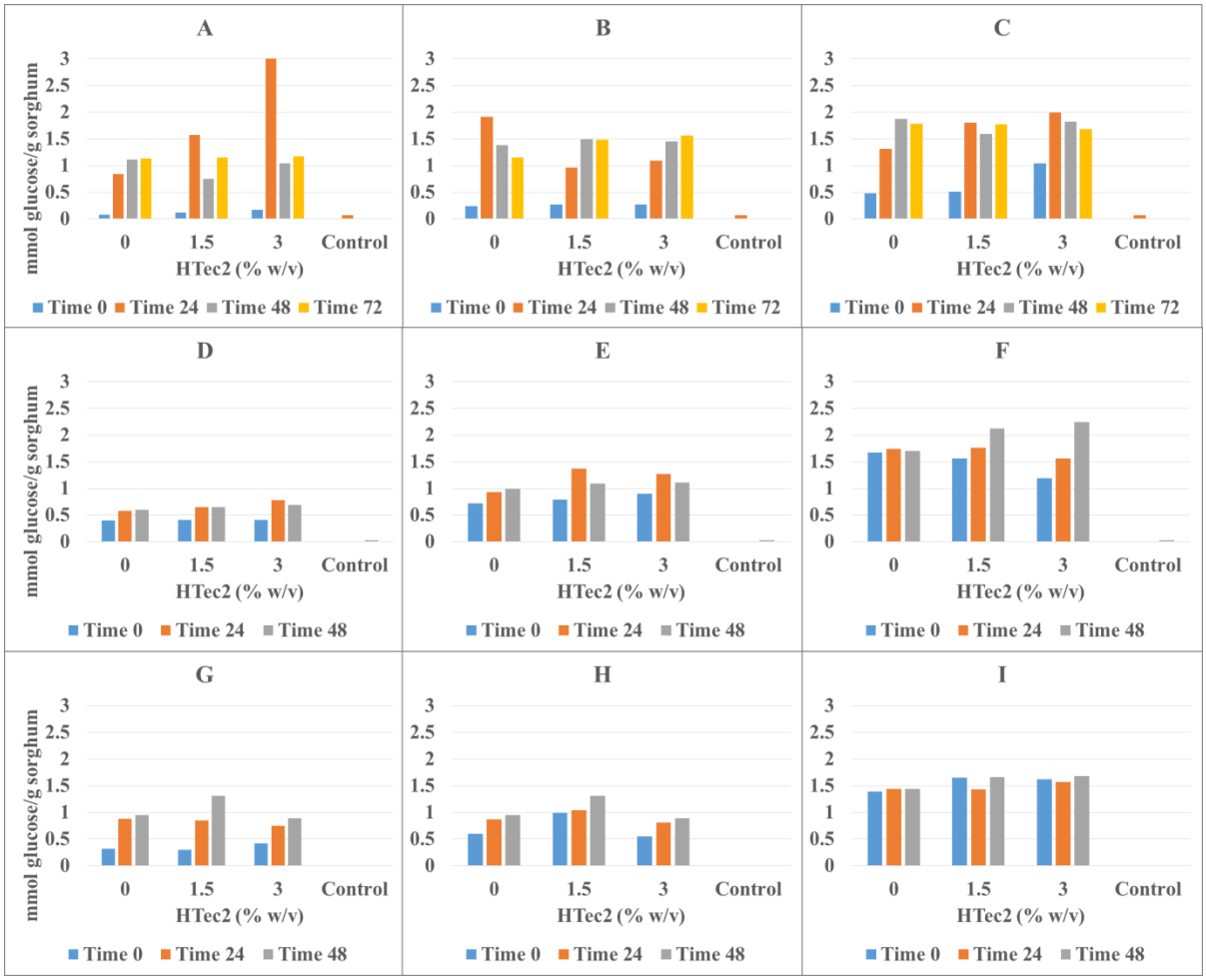

Figure 3.1: Glucose concentrations for ensiled sorghum at different levels of HTec2 at each time for each preparation and CTec2 loading: (A) Autoclaved CTec2 10 FPU/g (B) Autoclaved CTec2 25 FPU/g (C) Autoclaved 50 FPU/g (D) LNSA CTec2 10 FPU/g (E) LNSA CTec2 25 FPU/g (F) LNSA CTec2 50 FPU/g (G) LN CTec2 10 FPU/g (H) LN CTec2 25 FPU/g (I) LN CTec2 50 FPU/g

Inclusion of HTec2 increased the amount of glucose released with HTec2 3% w/v resulting in the highest rate of glucose release at each CTec2 loading level for LNSA preparations (Figure 3.1 D, E, F). Peak glucose concentrations in the hydrolysate and glucose release rates were seen during the first 24 hours of hydrolysis with decreases occurring from 24 hours to 48 hours for CTec2 10 FPU/g sorghum and CTec2 25 FPU/g sorghum loadings. Within the CTec2 50 FPU/g sorghum loading, HTec2 1.5% w/v and 3%

w/v enzyme loading levels supported rapid enough glucose release that losses in glucose concentrations in the hydrolysate were not observed. It is also likely that LNSA preparations were effective at inhibiting proliferation of native microbial contaminants in the ensiled sorghum.

Ensiled sorghum treated with LN resulted in higher glucose release rates at the CTec2 10 FPU/g sorghum loading than LNSA preparations, however at CTec2 25 FPU/g sorghum and 50 FPU/g sorghum, glucose release rates were lower than those seen for LNSA t preparations (Figure 3.1 G, H, I). Additionally, glucose release rates for both LN and LNSA preparations were significantly lower than those seen for sterilized preparations likely due to the additional pretreatment effects of the autoclaving process altering the structure of the sorghum and increasing enzyme accessibility to cellulose and hemicellulose components. Within LN preparations, higher glucose concentrations in the hydrolysate were observed for higher CTec2 loading levels and within each CTec2 loading level, increases in HTec2 loading levels further increased the amount of glucose released during hydrolysis. These results were similar to LNSA preparation results. Aside from the differences in the rate at which glucose was released in LN preparations at different CTec2 levels, maximum glucose concentrations in the hydrolysate were observed after 48 hours.

The main effects CTec2, HTec2, Treatment and Time were significant for xylose (p-value<0.05). The CTec2*HTec2*Treatment*Time (p-value<0.05) interaction was also significant for the xylose concentration in the hydrolysate suggesting that enzyme combinations and material preparations had an effect on xylose release rates of ensiled sorghums. Higher xylose concentrations in the hydrolysate were observed with increases in

CTec2 loading rates in sterilized preparations (autoclaved) (Figure 3.2) with the highest xylose concentrations in the hydrolysate occurring between 0 and 48 hours and no statistical differences between the amount of xylose released at 48 and 72 hours. Although increased CTec2 loading resulted in increased xylose release rate, HTec2 loading levels did not appear to have a significant effect on the amount of xylose released for sterilized preparations within a given CTec2 loading and over time.

For LNSA preparations at low CTec2 loading levels (CTec2 10 FPU/g sorghum) xylose concentrations in the hydrolysate increased from time 0 to time 24. However, at higher CTec2 loading levels (CTec2 50 FPU/g sorghum) xylose concentrations in the hydrolysate decreased from time 0 to time 24 but increased from time 24 to time 48 for all HTec2 levels (Figure 3.2 D,E,F) but remained lower than the initial xylose concentration in the hydrolysate. Similar to hydrolysis of sterilized material, increases in CTec2 loading resulted in increases in the initial xylose concentration in the hydrolysate, while increased HTec2 loading levels resulted in minimal differences in the initial xylose concentration in the hydrolysate in LNSA treated sorghum hydrolysis systems. Interestingly, decreases in xylose concentration in the hydrolysate from time 0 to time 48 were observed for higher CTec2 loading levels (25 FPU/g sorghum, 50 FPU/g sorghum) included HTec2 in hydrolysis. This loss was not observed at CTec2 25 FPU/g, 0% w/v HTec2 or preparations with the CTec2 10 FPU/g sorghum loading levels.

Xylose concentration in the hydrolysate of LN treated ensiled sorghum decreased from time 0 to time 24 hours for all enzyme combinations with slight increases occurring by 48 hours (Figure 3.2 G,H,I). Again the effect of the HTec2 loading levels on the amount of

xylose released was not as apparent as the impact of the increased CTec2 loading levels for the LN preparations. Initial xylose concentrations in the hydrolysate were the highest of any of the time steps suggesting that xylose was being utilized by contaminant microflora to produce byproducts during the hydrolysis process. Furthermore, no xylose was observed in the controls (no enzyme addition) for LN preparations indicating that initial xylose concentrations in the hydrolysate were the result of the enzyme formulation rather than the material itself. LNSA preparations outperformed LN preparations in terms of xylose released where the sodium azide may be complementing the lysozyme-nisin in inhibiting microbial activity.

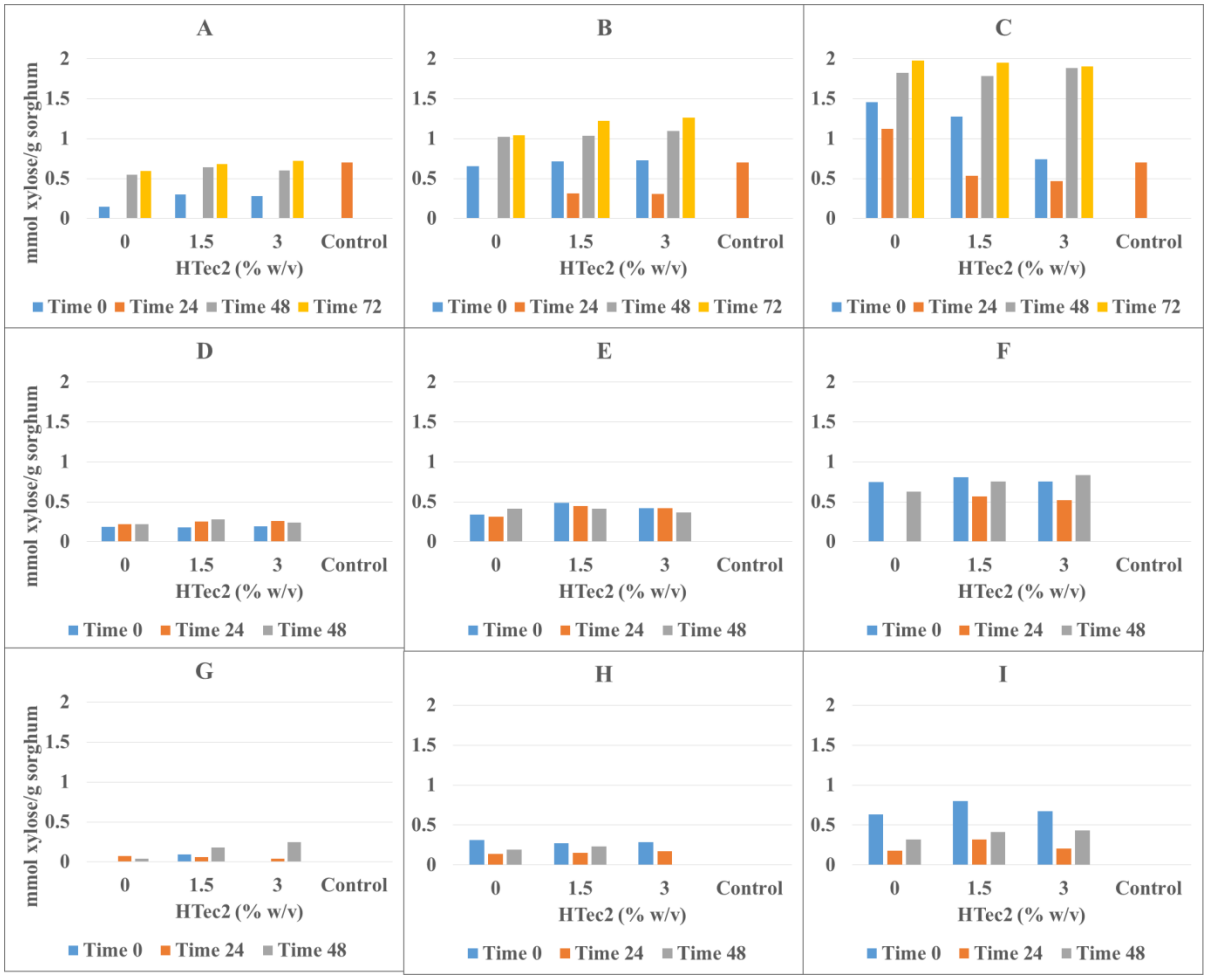

Figure 3.2: Xylose concentrations for ensiled sorghum at different levels of HTec2 at each time for each preparation and CTec2 loading: (A) Autoclaved CTec2 10 FPU/g (B) Autoclaved CTec2 25 FPU/g (C) Autoclaved 50 FPU/g (D) LNSA CTec2 10 FPU/g (E) LNSA CTec2 25 FPU/g (F) LNSA CTec2 50 FPU/g (G) LN CTec2 10 FPU/g (H) LN CTec2 25 FPU/g (I) LN CTec2 50 FPU/g

The main effects of Treatment and Time were significant for cellobiose and arabinose release (p-value<0.05) while the main effect of CTec2 was significant (p-value<0.05) for cellobiose alone. The CTec2*Treatment*Time interactions was also significant (p-value<0.05) for both cellobiose and arabinose suggesting that CTec2 loading influenced the rate at which those sugars were being released within each material preparation. Sterilized preparations had higher cellobiose and arabinose concentrations in the hydrolysate than LN

and LNSA preparations suggesting that the cellulase converted a greater fraction of the ensiled sorghum to glucose based on the combined higher concentrations of all sugar end products in the hydrolysate. Differences in cellobiose concentrations in the hydrolysate observed between LN and LNSA preparations were statistically similar. Cellobiose release rate was similar across CTec2 levels, with slight increases seen after 48 hours for CTec2 50 FPU/g for hydrolysis of sterilized material.

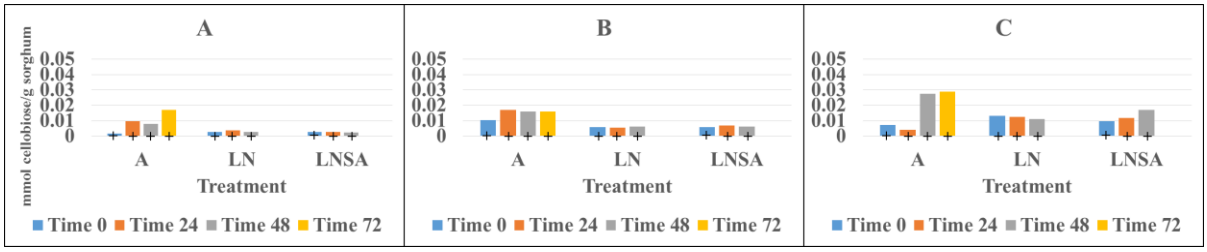

Figure 3.3: Cellobiose concentrations for ensiled sorghum for different material preparations (A, LN, LNSA) at each time for each CTec2 loading across HTec2: (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g (C) 50 FPU/g; (+) symbol represents no enzyme controls

The concentration of arabinose in the hydrolysate and the rate at which it was released from the substrate increased with CTec2 loading rate. Highest arabinose release rates occurred in the first 24 hours of hydrolysis with smaller increases occurring in the following 48 hours. Interestingly, LN and LNSA showed higher amounts of arabinose released at low CTec2 loading (10 FPU/g) indicating that some inhibition between preparation and CTec2 loading may be taking place.

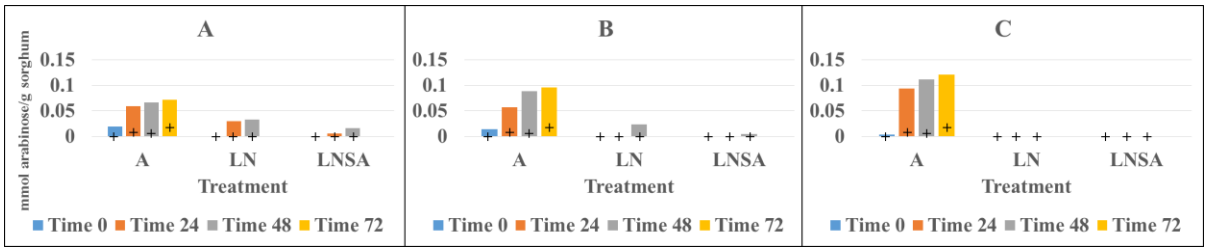

Figure 3.4: Arabinose concentrations for ensiled sorghum for different material preparations (A, LN, LNSA) at each time for each CTec2 loading across HTec2: (A) CTec2 10 FPU/g (B) CTec2 25 FPU/g (C) 50 FPU/g; (+) symbol represents no enzyme controls

Treatment and time main effects were significant for the formation of total byproducts (lactate, acetate and ethanol; p-value<0.05). The Treatment*Time interaction was also significant (p-value<0.05) for total byproduct formed suggesting that material preparation directly impacted the rate at which free sugars in the hydrolysate were being utilized to form acetate, lactate and ethanol by proliferating microflora during hydrolysis. Increases in the total byproduct concentration in the hydrolysate were observed in sterilized preparations from time 0 to 24 hours with statistically similar concentrations in the hydrolysate being maintained after through 72 hours (Figure 3.5). In comparison, LN and LNSA preparations showed no significant increases in concentration of total byproducts in the hydrolysate during the course of hydrolysis with slight decreases being shown during the first 24 hours for LN preparations. Although it was assumed that autoclaving as a sterilization technique would result in sterile material, sporulation by bacteria during the ensiling process due to low pH conditions may have released spores activated by the heat and pressure in the autoclaving process, leading to issues with microbials utilizing free sugars released during enzymatic hydrolysis to form byproducts. Sodium azide appeared to have

little effect on preventing byproduct formation as there was little difference in the byproduct formation rate between LN and LNSA preparations.

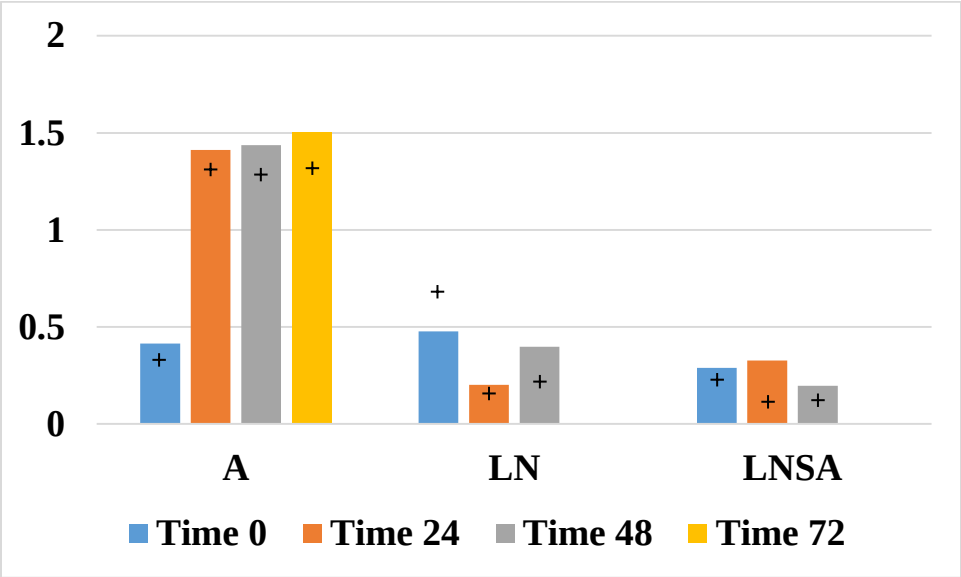

Figure 3.5: Total byproduct concentration for each material preparation (A, LN, LNSA) at each time across CTec2 and HTec2; (+) symbol represents no enzyme control

3.3.2 Enzyme and Cellulose to Glucose Conversion Efficiency

Differences in enzyme effectiveness, aside from the overall sugars released, were observed between the different treatment combinations (Table 3-3). No significant difference (p-value>0.05) in enzyme efficiency was observed for non-sterilized preparations (LN, LNSA) regardless of CTec2 loading except for at CTec2 50 FPU/g where LN was significantly (p-value<0.05) lower than LNSA. At lower enzyme loading (CTec2 10 FPU/g sorghum) maximum enzyme efficiency occurred in the first 24 hours, on average, while at higher CTec2 loadings (25 FPU/g sorghum, 50 FPU/g sorghum) maximum enzyme efficiency occurred after 48 hours except for LNSA at CTec2 25 FPU/g sorghum, suggesting

that the enzyme-substrate ratio impacts overall enzyme activity. Enzyme efficiency decreased significantly (p-value<0.05) when CTec2 loading was greater than 10 FPU/g sorghum for all material preparations but no statistical difference was observed between enzyme efficiency at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum. Differences in enzyme effectiveness are likely a result of substrate-enzyme interactions which can be influenced by substrate concentration and structure, free sugar concentration and pH (Sun and Cheng, 2002). Sattler et al (1988) showed that during the first 24 hours of a hydrolysis study on softwoods increased enzyme loading beyond 10 FPU/g dry weight resulted in negligible increases in glucose released. However, after the initial hydrolysis had occurred, increased enzyme loading resulted in increased glucose release with 10% more glucose being released at 25 FPU/g dry weight than at 10 FPU/g dry weight. Furthermore, Gregg and Saddler (1996) suggested that there was an inverse relationship between substrate concentration and glucose yield during hydrolysis meaning that at lower enzyme loading there is less activity beyond 24 hours than at higher enzyme loading rates. As seen in Table 3-4 maximum cellulose to glucose yields for CTec2 10 FPU/g were observed during the first 24 hours of hydrolysis while at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum maximum glucose yields were observed after 48 hours except for LNSA treatments at CTec2 25 FPU/g sorghum. Similar findings were determined in this work (Table 3-4) where in non-sterilized preparations (LN, LNSA) maximum cellulose to glucose conversion at CTec2 25 FPU/g sorghum and CTec2 50 FPU/g sorghum occurred after 48 hours, except for LNSA at CTec2 25 FPU/g sorghum. However, the interactions causing this effect are still unclear.

Sterilized preparations resulted in significantly higher (p-value<0.05) enzyme efficiencies than non-sterilized preparations (Table 3-3) regardless of CTec2 loading. Increased enzyme activity for sterilized preparations may be more related to structural changes in the substrate during the autoclaving in combination with weak acid pretreatment from ensiling (Linden et al., 1986; Cao et al., 2012). The substrate change likely allowed the enzymes to more easily access the substrate and increased the susceptibility of the cellulosic substrate to enzymatic hydrolysis (Gregg and Saddler, 1996). Sinitsyn et al (1991) suggested that increased surface area of the substrate rather than a lower proportion of crystalline material in the substrate was the cause for higher hydrolysis rates in cotton linter. This would also be true for the ensiled sorghum used in this study. As seen in Table 3-3, sterilized preparations had significantly higher enzymatic efficiency than non-sterilized preparations within each CTec2 loading level as a result of increased surface area (i.e. increased binding sites) creating greater opportunities for the enzymes to interact with the substrate regardless of activity.

Table 3-3-3: Amount of glucose released on an enzyme activity basis (mg glucose/FPU) for CTec2 10 FPU/g, CTec2 25 FPU/g and CTec2 50 FPU/g for each treatment where different capital letters represent significantly different values within CTec2 loading level (10 FPU/g, 25 FPU/g, 50 FPU/g) and lowercase letters represent significantly different values within each material treatment (A, LN, LNSA, SA)

CTec2 Loading Level	Treatment	Range of Glucose Released (mg glucose/FPU)	Maximum Glucose Released mg glucose/FPU [Time]
10	A	3.8-7.6	7.6^{Aa} (24)
	LN	1.2-2.2	2.2^{Ba} (24)
	LNSA	1.1-1.2	1.2^{Ba} (24)
25	A	1.9-2.1	2.1^{Ab} (48)
	LN	0.3-0.6	0.6^{Bb} (48)
	LNSA	0.5-0.8	0.8^{Ba} (24)
50	A	0.9-1.0	1.0^{Ab} (48)
	LN	0-0.4	0.4^{Bb} (48)
	LNSA	0.2-0.5	0.5^{Cb} (48)

Due to the increased accessibility of the substrate in sterilized preparations, the overall net cellulose to glucose conversion efficiencies of sterilized preparations was higher than non-sterilized preparations (LN, LNSA) regardless of CTec2 loading, as seen in Table 3-4. Despite lower enzyme activity due to the presence of lactate, changes in the substrate during autoclaving allowed higher cellulose to glucose conversion efficiency than non-sterilized preparations. Preparations that included LN and LNSA during hydrolysis of ensiled sorghum resulted in considerably lower net conversion efficiencies suggesting an inhibiting interaction between the additives, enzymes and substrate. The exact mechanism is not understood, however, the anticipated benefit of the additives working to minimize microbial proliferation of gram-positive bacteria was not realized, nor does it appear

necessary. Although 67.5% of cellulose was hydrolyzed to glucose it is clear that more cellulose was still available for conversion to glucose. Use of mild pretreatment on ensiled material should be investigated to increase cellulose to glucose conversion efficiency.

Table 3-4: Net Percentage of Cellulose that was Hydrolyzed to Glucose for Ensiled AF7401 Sorghum in Whole Sorghum Stalk Hydrolysis across HTec2 and Time for A, LN, LNSA, SA Treatments

Treatment	CTec2 (FPU/g w.w.)	Maximum Cellulose to Glucose Conversion (%) (hour)
A	10	67.5 (24)
	25	47.3 (48)
	50	43.4 (48)
LN	10	19.5 (24)
	25	13.6 (48)
	50	1.6 (48)
LNSA	10	10.7 (24)
	25	15.5 (24)
	50	22.2 (48)

Note: Values in Table 3-4 do not account for losses in glucose due to byproduct formation by microbial proliferation and represent a lower limit of conversion. Capital letters are comparison within treatments between loading lowercase within loading between treatments

3.4 Conclusion

Use of additives did not appear to provide any added benefit because ensiling lowered the pH to a point that proliferation of naturally occurring microflora appeared to be limited. Increases in enzyme loading within this sorghum hydrolysis system did not improve enzyme efficiency or extent or net conversion. In situations where sterilization is critical the use of lactic or acetic acid could mimic sterilization without decreasing the activity of cellulase enzymes during hydrolysis. The potential benefit of adding HTec2 to the enzyme cocktail for ensiled sorghum was not realized and additional cost analysis may be necessary to fully

justify its addition. Ensilage as a storage method prior to enzymatic hydrolysis seems promising.

3.5 References

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APPENDICES

Appendix A: SAS Analysis Effects of Enzyme Loading, Material Treatment and Time on Dried Sorghums

Appendix A.1 Glucose

SAS Code

```
proc glm data=sugar;
  where material="Dried" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model glucose = CTec2|HTec2|treatment|time;
  run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	107	54.80946943	0.51223803	35.22	<.0001
Error	216	3.14171721	0.01454499		
Corrected Total	323	57.95118663			

R-Square	Coeff Var	Root MSE	Glucose Mean
0.945787	22.23291	0.120603	0.542451

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	4.21812774	2.10906387	145.00	<.0001
HTec2	2	0.14396825	0.07198412	4.95	0.0079
CTec2*HTec2	4	0.11746260	0.02936565	2.02	0.0929
treatment	3	31.33979103	10.44659701	718.23	<.0001
CTec2*treatment	6	0.80837092	0.13472849	9.26	<.0001
HTec2*treatment	6	0.10867155	0.01811193	1.25	0.2844
CTec2*HTec2*treatmen	12	0.49956650	0.04163054	2.86	0.0011
Time	2	11.64697985	5.82348993	400.38	<.0001
CTec2*Time	4	0.67371551	0.16842888	11.58	<.0001
HTec2*Time	4	0.08798753	0.02199688	1.51	0.1996
CTec2*HTec2*Time	8	0.10077097	0.01259637	0.87	0.5460
Time*treatment	6	4.20436687	0.70072781	48.18	<.0001
CTec2*Time*treatment	12	0.33607029	0.02800586	1.93	0.0328
HTec2*Time*treatment	12	0.18875569	0.01572964	1.08	0.3770
CTec*HTec*Time*treat	24	0.33486412	0.01395267	0.96	0.5215

Appendix A.2 Xylose

SAS Code

```
proc glm data=sugar;
  where material="Dried" and time in (0,24,48) and CTec2 in("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model  = CTec2|HTec2|treatment|time;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	107	11.86499972	0.11088785	20.57	<.0001
Error	216	1.16412101	0.00538945		
Corrected Total	323	13.02912073			

R-Square	Coeff Var	Root MSE	Xylose Mean
0.910652	24.82528	0.073413	0.295718

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	2.63014285	1.31507142	244.01	<.0001
HTec2	2	0.08694620	0.04347310	8.07	0.0004
CTec2*HTec2	4	0.03358618	0.00839654	1.56	0.1867
treatment	3	5.71217203	1.90405734	353.29	<.0001
CTec2*treatment	6	0.97172195	0.16195366	30.05	<.0001
HTec2*treatment	6	0.02976038	0.00496006	0.92	0.4811
CTec2*HTec2*treatmen	12	0.11648892	0.00970741	1.80	0.0494
Time	2	1.03983400	0.51991700	96.47	<.0001
CTec2*Time	4	0.08769948	0.02192487	4.07	0.0034
HTec2*Time	4	0.04513520	0.01128380	2.09	0.0827
CTec2*HTec2*Time	8	0.03018319	0.00377290	0.70	0.6913
Time*treatment	6	0.57771306	0.09628551	17.87	<.0001
CTec2*Time*treatment	12	0.36497797	0.03041483	5.64	<.0001
HTec2*Time*treatment	12	0.04333750	0.00361146	0.67	0.7791
CTec*HTec*Time*treat	24	0.09530081	0.00397087	0.74	0.8103

Appendix A.3 Cellobiose

SAS Code

```
proc glm data=sugar;
  where material="Dried" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model cellobiose1  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	107	0.33782445	0.00315724	13.92	<.0001
Error	216	0.04899738	0.00022684		
Corrected Total	323	0.38682183			

R-Square	Coeff Var	Root MSE	Cellobiose1 Mean
0.873333	94.67251	0.015061	0.015909

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.02844191	0.01422096	62.69	<.0001
HTec2	2	0.00009537	0.00004768	0.21	0.8106
CTec2*HTec2	4	0.00522988	0.00130747	5.76	0.0002
treatment	3	0.10720859	0.03573620	157.54	<.0001
CTec2*treatment	6	0.06358494	0.01059749	46.72	<.0001
HTec2*treatment	6	0.00109091	0.00018182	0.80	0.5697
CTec2*HTec2*treatmen	12	0.00979064	0.00081589	3.60	<.0001
Time	2	0.01515401	0.00757700	33.40	<.0001
CTec2*Time	4	0.01400334	0.00350083	15.43	<.0001
HTec2*Time	4	0.00038146	0.00009536	0.42	0.7938
CTec2*HTec2*Time	8	0.00914943	0.00114368	5.04	<.0001
Time*treatment	6	0.02435917	0.00405986	17.90	<.0001
CTec2*Time*treatment	12	0.02862704	0.00238559	10.52	<.0001
HTec2*Time*treatment	12	0.00147380	0.00012282	0.54	0.8860
CTec*HTec*Time*treat	24	0.02923396	0.00121808	5.37	<.0001

Appendix A.4 Arabinose

SAS Code

```
proc glm data=sugar;
where material="Dried" and time in (0,24,48) and CTec2 in ("10","25","50");
class  CTec2 HTec2 time treatment;
model arabinose  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	107	0.11752026	0.00109832	37.21	<.0001
Error	216	0.00637487	0.00002951		
Corrected Total	323	0.12389514			

R-Square	Coeff Var	Root MSE	Arabinose Mean
0.948546	27.02812	0.005433	0.020100

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.00466212	0.00233106	78.98	<.0001
HTec2	2	0.00013615	0.00006807	2.31	0.1021
CTec2*HTec2	4	0.00006156	0.00001539	0.52	0.7200
treatment	3	0.02466749	0.00822250	278.60	<.0001
CTec2*treatment	6	0.00383167	0.00063861	21.64	<.0001
HTec2*treatment	6	0.00025210	0.00004202	1.42	0.2067
CTec2*HTec2*treatmen	12	0.00086277	0.00007190	2.44	0.0055
Time	2	0.06255290	0.03127645	1059.74	<.0001
CTec2*Time	4	0.00330582	0.00082646	28.00	<.0001
HTec2*Time	4	0.00011480	0.00002870	0.97	0.4235
CTec2*HTec2*Time	8	0.00012251	0.00001531	0.52	0.8416
Time*treatment	6	0.01278361	0.00213060	72.19	<.0001
CTec2*Time*treatment	12	0.00266931	0.00022244	7.54	<.0001
HTec2*Time*treatment	12	0.00037487	0.00003124	1.06	0.3969
CTec*HTec*Time*treat	24	0.00112258	0.00004677	1.58	0.0460

Appendix A.5 Total Byproducts

SAS Code

```
proc glm data=sugar;
  where material="Dried" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model totalbyproducts  = CTec2|HTec2|treatment|time;;;
        run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	107	15.28823060	0.14288066	13.07	<.0001
Error	216	2.36207540	0.01093553		
Corrected Total	323	17.65030600			

R-Square	Coeff Var	Root MSE	TotalByproducts Mean
0.866174	43.77726	0.104573	0.238875

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.02747702	0.01373851	1.26	0.2868
HTec2	2	0.03095634	0.01547817	1.42	0.2451
CTec2*HTec2	4	0.03810773	0.00952693	0.87	0.4820
treatment	3	3.53429375	1.17809792	107.73	<.0001
CTec2*treatment	6	0.08212684	0.01368781	1.25	0.2812
HTec2*treatment	6	0.08239867	0.01373311	1.26	0.2792
CTec2*HTec2*treatmen	12	0.12931739	0.01077645	0.99	0.4638
Time	2	5.75052233	2.87526116	262.93	<.0001
CTec2*Time	4	0.05089405	0.01272351	1.16	0.3279
HTec2*Time	4	0.07194785	0.01798696	1.64	0.1641
CTec2*HTec2*Time	8	0.10281083	0.01285135	1.18	0.3153
Time*treatment	6	4.72159223	0.78693204	71.96	<.0001
CTec2*Time*treatment	12	0.15851934	0.01320994	1.21	0.2788
HTec2*Time*treatment	12	0.19965059	0.01663755	1.52	0.1179
CTec*HTec*Time*treat	24	0.30761564	0.01281732	1.17	0.2702

Appendix B: SAS Analysis Effects of Enzyme Loading, Material Treatment and Time on Fresh Sorghums

Appendix B.1 Glucose

SAS Code

```
proc glm data=sugar;
  where material="Fresh" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model cellobiose1  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects:

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	60.36286960	0.75453587	19.75	<.0001
Error	162	6.19016627	0.03821090		
Corrected Total	242	66.55303587			

R-Square	Coeff Var	Root MSE	Glucose Mean
0.906989	27.44983	0.195476	0.712121

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	14.98053816	7.49026908	196.02	<.0001
HTec2	2	0.40527655	0.20263828	5.30	0.0059
CTec2*HTec2	4	0.14098331	0.03524583	0.92	0.4524
treatment	2	3.24731562	1.62365781	42.49	<.0001
CTec2*treatment	4	1.32178584	0.33044646	8.65	<.0001
HTec2*treatment	4	0.52151855	0.13037964	3.41	0.0104
CTec2*HTec2*treatmen	8	0.29165696	0.03645712	0.95	0.4740
Time	2	22.60156970	11.30078485	295.75	<.0001
CTec2*Time	4	4.20556910	1.05139227	27.52	<.0001
HTec2*Time	4	0.29176973	0.07294243	1.91	0.1114
CTec2*HTec2*Time	8	0.22244545	0.02780568	0.73	0.6669
treatment*Time	4	9.12876246	2.28219061	59.73	<.0001
CTec2*treatment*Time	8	1.08965344	0.13620668	3.56	0.0008
HTec2*treatment*Time	8	0.68393348	0.08549168	2.24	0.0273
CTec*HTec*treat*Time	16	1.23009126	0.07688070	2.01	0.0151

Appendix B.2 Xylose

SAS Code

```
proc glm data=sugar;
  where material="Fresh" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model xylose  = CTec2|HTec2|treatment|time;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	18.84043860	0.23550548	49.81	<.0001
Error	162	0.76598404	0.00472830		
Corrected Total	242	19.60642264			

R-Square	Coeff Var	Root MSE	Xylose Mean
0.960932	14.20769	0.068763	0.483982

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	9.68976858	4.84488429	1024.66	<.0001
HTec2	2	0.02021749	0.01010874	2.14	0.1212
CTec2*HTec2	4	0.15361419	0.03840355	8.12	<.0001
treatment	2	4.38943510	2.19471755	464.17	<.0001
CTec2*treatment	4	1.32389593	0.33097398	70.00	<.0001
HTec2*treatment	4	0.02825938	0.00706484	1.49	0.2064
CTec2*HTec2*treatmen	8	0.07556874	0.00944609	2.00	0.0498
Time	2	0.29002833	0.14501417	30.67	<.0001
CTec2*Time	4	0.36171986	0.09042997	19.13	<.0001
HTec2*Time	4	0.01367689	0.00341922	0.72	0.5773
CTec2*HTec2*Time	8	0.10251892	0.01281487	2.71	0.0079
treatment*Time	4	1.75199626	0.43799906	92.63	<.0001
CTec2*treatment*Time	8	0.29052863	0.03631608	7.68	<.0001
HTec2*treatment*Time	8	0.06099703	0.00762463	1.61	0.1249
CTec*HTec*treat*Time	16	0.28821327	0.01801333	3.81	<.0001

Appendix B.3 Cellobiose

SAS Code

```
proc glm data=sugar;
  where material="Fresh" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model cellobiose1  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	0.01146554	0.00014332	6.62	<.0001
Error	162	0.00350597	0.00002164		
Corrected Total	242	0.01497151			

R-Square	Coeff Var	Root MSE	Cellobiose1 Mean
0.765824	51.66321	0.004652	0.009005

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.00579635	0.00289817	133.92	<.0001
HTec2	2	0.00003168	0.00001584	0.73	0.4825
CTec2*HTec2	4	0.00002584	0.00000646	0.30	0.8786
treatment	2	0.00044059	0.00022029	10.18	<.0001
CTec2*treatment	4	0.00091638	0.00022909	10.59	<.0001
HTec2*treatment	4	0.00004133	0.00001033	0.48	0.7522
CTec2*HTec2*treatmen	8	0.00017689	0.00002211	1.02	0.4218
Time	2	0.00090468	0.00045234	20.90	<.0001
CTec2*Time	4	0.00049978	0.00012495	5.77	0.0002
HTec2*Time	4	0.00006400	0.00001600	0.74	0.5664
CTec2*HTec2*Time	8	0.00031681	0.00003960	1.83	0.0750
treatment*Time	4	0.00028960	0.00007240	3.35	0.0116
CTec2*treatment*Time	8	0.00149248	0.00018656	8.62	<.0001
HTec2*treatment*Time	8	0.00023523	0.00002940	1.36	0.2185
CTec*HTec*treat*Time	16	0.00023387	0.00001462	0.68	0.8152

Appendix B.4 Arabinose

SAS Code

```
proc glm data=sugar;
   where material="Fresh" and time in (0,24,48) and CTec2 in ("10","25","50");
   class  CTec2 HTec2 time treatment ;
   model arabinose  = CTec2|HTec2|treatment|time;;;
run;
```

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	0.10945203	0.00136815	6.70	<.0001
Error	162	0.03306959	0.00020413		
Corrected Total	242	0.14252162			

R-Square	Coeff Var	Root MSE	Arabinose Mean
0.767968	57.38189	0.014288	0.024899

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.00310843	0.00155421	7.61	0.0007
HTec2	2	0.00004749	0.00002375	0.12	0.8903
CTec2*HTec2	4	0.00079180	0.00019795	0.97	0.4258
treatment	2	0.00157550	0.00078775	3.86	0.0231
CTec2*treatment	4	0.00418229	0.00104557	5.12	0.0007
HTec2*treatment	4	0.00103145	0.00025786	1.26	0.2867
CTec2*HTec2*treatmen	8	0.00120331	0.00015041	0.74	0.6588
Time	2	0.07482712	0.03741356	183.28	<.0001
CTec2*Time	4	0.00833249	0.00208312	10.20	<.0001
HTec2*Time	4	0.00038539	0.00009635	0.47	0.7562
CTec2*HTec2*Time	8	0.00076640	0.00009580	0.47	0.8764
treatment*Time	4	0.00361918	0.00090480	4.43	0.0020
CTec2*treatment*Time	8	0.00532370	0.00066546	3.26	0.0018
HTec2*treatment*Time	8	0.00115752	0.00014469	0.71	0.6835
CTec*HTec*treat*Time	16	0.00309997	0.00019375	0.95	0.5149

Appendix B.5 Total Byproducts

SAS Code

```
proc glm data=sugar;
  where material="Fresh" and time in (0,24,48) and CTec2 in ("10","25","50");
  class  CTec2 HTec2 time treatment ;
  model totalbyproducts  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	17.66172750	0.22077159	5.99	<.0001
Error	162	5.96766337	0.03683743		
Corrected Total	242	23.62939087			

R-Square	Coeff Var	Root MSE	TotalByproducts Mean
0.747447	88.92222	0.191931	0.215841

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.14406878	0.07203439	1.96	0.1448
HTec2	2	0.03790145	0.01895073	0.51	0.5988
CTec2*HTec2	4	0.35611685	0.08902921	2.42	0.0508
treatment	2	4.46090030	2.23045015	60.55	<.0001
CTec2*treatment	4	0.01702398	0.00425600	0.12	0.9769
HTec2*treatment	4	0.09129179	0.02282295	0.62	0.6492
CTec2*HTec2*treatmen	8	0.23157763	0.02894720	0.79	0.6158
Time	2	3.80479731	1.90239865	51.64	<.0001
CTec2*Time	4	0.39888600	0.09972150	2.71	0.0322
HTec2*Time	4	0.06789955	0.01697489	0.46	0.7644
CTec2*HTec2*Time	8	0.77300438	0.09662555	2.62	0.0100
treatment*Time	4	6.31447174	1.57861793	42.85	<.0001
CTec2*treatment*Time	8	0.08411693	0.01051462	0.29	0.9700
HTec2*treatment*Time	8	0.10160071	0.01270009	0.34	0.9471
CTec*HTec*treat*Time	16	0.77807012	0.04862938	1.32	0.1905

Appendix C: SAS Analysis Effects of Enzyme Loading, Material Treatment and Time on Dried Sorghums

Appendix C.1 Glucose

SAS Code

```
proc glm data=sugar;
  where material="Ensiled" and time in (0,24,48) and CTec2 in ("10","25","50") and
treatment in ("A","LN","LNSA");
  class  CTec2 HTec2 time treatment ;
  model glucose  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	78.83281201	0.98541015	20.56	<.0001
Error	162	7.76575987	0.04793679		
Corrected Total	242	86.59857188			

R-Square	Coeff Var	Root MSE	Glucose Mean
0.910325	20.64406	0.218945	1.060570

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	30.57514358	15.28757179	318.91	<.0001
HTec2	2	2.07572748	1.03786374	21.65	<.0001
CTec2*HTec2	4	0.76874928	0.19218732	4.01	0.0040
treatment	2	0.28739316	0.14369658	3.00	0.0527
CTec2*treatment	4	4.37090277	1.09272569	22.80	<.0001
HTec2*treatment	4	1.97556123	0.49389031	10.30	<.0001
CTec2*HTec2*treatmen	8	0.75531790	0.09441474	1.97	0.0534
Time	2	15.38856971	7.69428485	160.51	<.0001
CTec2*Time	4	2.94857323	0.73714331	15.38	<.0001
HTec2*Time	4	1.22730039	0.30682510	6.40	<.0001
CTec2*HTec2*Time	8	0.73265496	0.09158187	1.91	0.0617
Time*treatment	4	7.19130087	1.79782522	37.50	<.0001
CTec2*Time*treatment	8	4.68588339	0.58573542	12.22	<.0001
HTec2*Time*treatment	8	3.69347495	0.46168437	9.63	<.0001
CTec*HTec*Time*treat	16	2.15625909	0.13476619	2.81	0.0005

Appendix C.2 Xylose

SAS Code

```
proc glm data=sugar;
        where material="Ensiled" and time in (0,24,48) and CTec2 in
("10","25","50") and treatment in ("A","LN","LNSA");
 class  CTec2 HTec2 time treatment ;
model xylose  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects The SAS System

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	144.8122100	1.8101526	43.88	<.0001
Error	162	6.6822726	0.0412486		
Corrected Total	242	151.4944826			

R-Square	Coeff Var	Root MSE	Xylose Mean
0.955891	21.39824	0.203098	0.949132

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	23.46448946	11.73224473	284.43	<.0001
HTec2	2	0.44135809	0.22067905	5.35	0.0056
CTec2*HTec2	4	0.14710739	0.03677685	0.89	0.4704
treatment	2	65.01367566	32.50683783	788.07	<.0001
CTec2*treatment	4	7.59730329	1.89932582	46.05	<.0001
HTec2*treatment	4	1.39848325	0.34962081	8.48	<.0001
CTec2*HTec2*treatmen	8	0.77788642	0.09723580	2.36	0.0200
Time	2	9.48210929	4.74105465	114.94	<.0001
CTec2*Time	4	1.00846182	0.25211545	6.11	0.0001
HTec2*Time	4	1.10694807	0.27673702	6.71	<.0001
CTec2*HTec2*Time	8	1.04951429	0.13118929	3.18	0.0022
Time*treatment	4	24.79045279	6.19761320	150.25	<.0001
CTec2*Time*treatment	8	5.39483051	0.67435381	16.35	<.0001
HTec2*Time*treatment	8	1.46098893	0.18262362	4.43	<.0001
CTec*HTec*Time*treat	16	1.67860075	0.10491255	2.54	0.0016

Appendix C.3 Cellobiose

SAS Code

```
proc glm data=sugar;
  where material="Ensiled" and time in (0,24,48) and CTec2 in ("10","25","50") and
treatment in ("A","LN","LNSA");
  class  CTec2 HTec2 time treatment ;
  model cellobiose1  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	0.01017502	0.00012719	3.63	<.0001
Error	162	0.00568201	0.00003507		
Corrected Total	242	0.01585703			

R-Square	Coeff Var	Root MSE	Cellobiose1 Mean
0.641672	69.60727	0.005922	0.008508

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.00305249	0.00152624	43.51	<.0001
HTec2	2	0.00006920	0.00003460	0.99	0.3751
CTec2*HTec2	4	0.00003985	0.00000996	0.28	0.8880
treatment	2	0.00091094	0.00045547	12.99	<.0001
CTec2*treatment	4	0.00061049	0.00015262	4.35	0.0023
HTec2*treatment	4	0.00023789	0.00005947	1.70	0.1535
CTec2*HTec2*treatmen	8	0.00020768	0.00002596	0.74	0.6559
Time	2	0.00074072	0.00037036	10.56	<.0001
CTec2*Time	4	0.00091141	0.00022785	6.50	<.0001
HTec2*Time	4	0.00016376	0.00004094	1.17	0.3273
CTec2*HTec2*Time	8	0.00023570	0.00002946	0.84	0.5687
Time*treatment	4	0.00093262	0.00023315	6.65	<.0001
CTec2*Time*treatment	8	0.00115688	0.00014461	4.12	0.0002
HTec2*Time*treatment	8	0.00038786	0.00004848	1.38	0.2078
CTec*HTec*Time*treat	16	0.00051755	0.00003235	0.92	0.5451

Appendix C.4 Arabinose

SAS Code

```
proc glm data=sugar;
  where material="Ensiled" and time in (0,24,48) and CTec2 in ("10","25","50") and
treatment in ("A","LN","LNSA");
  class  CTec2 HTec2 time treatment ;
  model arabinose  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	0.28588502	0.00357356	30.94	<.0001
Error	162	0.01871167	0.00011550		
Corrected Total	242	0.30459669			

R-Square	Coeff Var	Root MSE	Arabinose Mean
0.938569	44.66057	0.010747	0.024064

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.00029421	0.00014710	1.27	0.2826
HTec2	2	0.00024228	0.00012114	1.05	0.3527
CTec2*HTec2	4	0.00090884	0.00022721	1.97	0.1020
treatment	2	0.15514109	0.07757055	671.58	<.0001
CTec2*treatment	4	0.01255609	0.00313902	27.18	<.0001
HTec2*treatment	4	0.00042481	0.00010620	0.92	0.4541
CTec2*HTec2*treatmen	8	0.00038775	0.00004847	0.42	0.9080
Time	2	0.05082047	0.02541023	219.99	<.0001
CTec2*Time	4	0.00075703	0.00018926	1.64	0.1670
HTec2*Time	4	0.00018570	0.00004643	0.40	0.8071
CTec2*HTec2*Time	8	0.00094643	0.00011830	1.02	0.4199
Time*treatment	4	0.04577913	0.01144478	99.09	<.0001
CTec2*Time*treatment	8	0.01417473	0.00177184	15.34	<.0001
HTec2*Time*treatment	8	0.00075023	0.00009378	0.81	0.5930
CTec*HTec*Time*treat	16	0.00251624	0.00015727	1.36	0.1671

Appendix C.5 Total Byproducts

SAS Code

```
proc glm data=sugar;
  where material="Ensiled" and time in (0,24,48) and CTec2 in ("10","25","50") and
treatment in ("A","LN","LNSA");
  class  CTec2 HTec2 time treatment ;
  model Totalbyproducts  = CTec2|HTec2|treatment|time;;;
run;
```

Main and Interaction Effects

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	80	72.5244920	0.9065561	3.87	<.0001
Error	162	37.9731718	0.2344023		
Corrected Total	242	110.4976638			

R-Square	Coeff Var	Root MSE	TotalByproducts Mean
0.656344	83.37710	0.484151	0.580676

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CTec2	2	0.61252096	0.30626048	1.31	0.2736
HTec2	2	0.24276306	0.12138153	0.52	0.5968
CTec2*HTec2	4	1.39833008	0.34958252	1.49	0.2072
treatment	2	32.71553932	16.35776966	69.79	<.0001
CTec2*treatment	4	1.22148697	0.30537174	1.30	0.2712
HTec2*treatment	4	1.44425061	0.36106265	1.54	0.1929
CTec2*HTec2*treatmen	8	1.73482500	0.21685313	0.93	0.4973
Time	2	4.39170460	2.19585230	9.37	0.0001
CTec2*Time	4	1.38138097	0.34534524	1.47	0.2127
HTec2*Time	4	1.20641092	0.30160273	1.29	0.2774
CTec2*HTec2*Time	8	2.02359128	0.25294891	1.08	0.3803
Time*treatment	4	15.23453542	3.80863385	16.25	<.0001
CTec2*Time*treatment	8	2.12787306	0.26598413	1.13	0.3427
HTec2*Time*treatment	8	1.58992914	0.19874114	0.85	0.5620
CTec*HTec*Time*treat	16	5.19935060	0.32495941	1.39	0.1542