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Title

Impact of various storage conditions on enzymatic activity, biomass components and conversion to ethanol yields from sorghum biomass used as a bioenergy crop

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Highlights

- Lignocellulosic biomass quality from harvest to ethanol production was monitored.
- Biomass was stored as bales covered or uncovered for up to six months.
- Covered bales maintained cellulose content, enzymatic activity and ethanol yields.
- Uncovered bales had lower cellulose, ethanol yields; increased enzymatic activity.
- Biomass coverage vital to maintain quality during storage for ethanol production.

Abstract

With increased mandates for biofuel production in the US, ethanol production from lignocellulosic substrates is burgeoning, highlighting the need for thorough examination of the biofuel production supply chain. This research focused on the impact storage has on biomass, particularly

Abbreviations: EEA, extra-cellular enzyme activity.

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photoperiod-sensitive sorghum biomass. Biomass quality parameters were monitored and included biomass components, cellulose, hemicellulose and lignin, along with extra-cellular enzymatic activity (EEA) responsible for cellulose and hemicellulose degradation and conversion to ethanol yields. Analyses revealed dramatic decreases in uncovered treatments, specifically reduced dry matter content from 88% to 59.9%, cellulose content from 35.3% to 25%, hemicellulose content from 23.7% to 16.0% and ethanol production of 0.20 g L^{-1} to 0.02 g L^{-1} after 6 months storage along with almost double EEA activities. In contrast, biomass components, EEA and ethanol yields remained relatively stable in covered treatments, indicating covering of biomass during storage is essential for optimal substrate retention and ethanol yields.

Key Words: lignocellulosic ethanol production; ethanol; biomass; storage; extra-cellular enzyme activity; biofuels; composition.

1. Introduction

In 2007, the United States government signed into action the Energy Independence and Security Act (EISA), which expanded the Renewable Fuel Standards (RFS) program and outlined government mandated goals for the utilization and production of renewable fuels in the United States by 2022. In 2010 the revised RFS2 mandates outlined that 136 billion liters of renewable fuels be used in the US and 60.5 of 136 billion liters be produced using the cellulosic platform or second generation technologies, along with goals for reduced greenhouse gas (GHG) emissions (Gao et al., 2011). Second generation biofuels are those produced from lignocellulosic materials, including agricultural and forestry residues, solid waste, perennial woody and herbaceous energy crops (Gibbons and Hughes, 2009). The driving factors of the economic competitiveness of cellulosic ethanol production are feedstock cost, availability, storage and transportation expenses (Hess et al., 2007). Estimates from Hess et al. (2007) value feedstock costs to be 35-50% of total ethanol production costs, with the logistics associated with moving

the biomass to the biorefinery comprising 50-75% of the feedstock costs. These high logistical costs reduce the potential profit margins for biomass producers and biorefinery operators and are therefore a serious concern (Hess et al., 2007).

A majority of the potential lignocellulosic feedstocks are harvested annually or bi-annually, resulting in extended storage to provide a continuous supply to a biorefinery. As a result, a large proportion of the biomass must be stored and preserved to provide a consistent, year-round feedstock supply to the biorefineries. Identifying the most cost effective method to minimize dry matter losses is essential. Minimizing dry matter loss during storage could play a key role in reducing the total biomass costs for the biomass producer and biorefinery operator and be crucial for the overall profitability of these operations. Biomass can be stored under wet or dry methods, both having advantages and disadvantages. Zheng et al. (2012) found that ensilage (wet storage) of sugar beet pulp (SBP) was an effective combined storage and pretreatment method that resulted in increased ethanol yields (0.2 g ethanol/g SBP) via fermentation with *E. coli* KO11. However, few studies have fully detailed the resulting ethanol yields from biomass stored under dry conditions over an extended period of time or monitored changes occurring during that storage period (Emery and Mosier, 2012).

Extra-cellular enzymes are the main mediators of soil biological processes, including organic matter degradation, mineralization and nutrient cycling (Marx et al., 2001). Substrate degradation and availability to microbial or plant uptake are believed to be controlled by hydrolytic enzymes (Marx et al., 2001). Monitoring enzymatic activity in stored biomass, specifically related to organic matter degradation, can be used as an indicator of biomass quality. Monitoring the enzymatic degradation of cellulose and hemicellose is of greatest interest for conversion of plant biomass to ethanol. A wide variety of methods have been developed for estimating enzyme activities in soil samples. These methods vary in substrate choices, assay conditions, incubation time and detection methods (e.g.,

colorimetric, fluorimetric, radiolabelled) (Marx et al., 2001). The fluorescent compound 4-methylumbelliferone (MUB) has great advantages over other enzyme assays and the assay principles have been previously described (Darrah and Harris 1996; Freeman et al., 1995; Hoppe 1983; Marx et al., 2001; Somville 1984). These advantages include high fluorescence of the MUB conjugate allowing minimal quantities to be detected, no reported inhibition or facilitation of the enzymatic activity, and measured enzymatic activity using MUB substrates is similar to the natural processes (Marx et al., 2001). The sensitivity of MUB-linked substrates also allows for the utilization of a 96-well plate format, which conveniently allows rapid measurement of activity for a range of enzymes and a large number of samples.

The goal of this study was to investigate the potential impact of four dry storage methods of sorghum biomass on the conversion to ethanol. We monitored the changes in biomass components, extra-cellular enzyme activity, and conversion to ethanol yields during each storage condition and duration. These studies critically evaluated the effect storage has on biomass components, including dry matter, cellulose, hemicellulose and lignin and enzymes specifically associated with cellulose and hemicellulose degradation.

2. Materials and Methods

All analyses were carried out as described below. After bale grinding, sub-samples for biomass component or conversion to ethanol and enzymatic activity analyses were immediately stored at -20°C or -80°C, respectively, until analysis.

2.1 Feedstock

Photo-period sensitive sorghum cultivar PS1990, a forage hybrid, from Sorghum Partners (Sorghum Partners, LLC, New Deal, TX) was grown on the North Agronomy Farm, Department of Agronomy, Kansas

State University in Manhattan, KS. The sorghum was planted on June 3, 2010. Nitrogen was applied 15 to 20 days after planting at a rate of 100 pounds per acre using urea (0-46-0) and herbicide Bicep II Magnum applied at 2.4 quarts per acre. Sorghum was cut and windrowed on October 7, 2010 and allowed to field dry. Cut and windrowed sorghum was baled into small square bales (average size 0.36 by 0.46 by 0.91 m) on October 15, 2010 using a Massey Ferguson (AGCO, Duluth, GA) square baler.

2.2 Storage Treatments

The bales were randomly assigned to one of four treatment groups: no plastic/no tarp (NN), no plastic/tarp (NT), plastic/no tarp (PN) and plastic/tarp (PT). All bales were assigned a random number from one to 96 and weighed. Those bales requiring plastic wrap were first wrapped two to three times with Tytan Wrap Premium Silage Film, 750 mm by 1500 m by 25.4 µm (Tytan International, LLC, Lenexa, KS). The PN and PT bales were then placed in two extra-large, industrial strength black plastic trash bags (Husky Brand, 45 gallon with 1mm thickness, Home Depot). The bales were randomly assigned to a sampling time point of zero, two, four or six months with six bales per treatment per sampling time. Bales were placed on wooden pallets in a single layer, grouped by sampling time point and covered with a tarp as needed (treatments NT and PT). Bales from each treatment and sampling time were arranged in the same configuration as well.

2.3 Bale Sampling

At sampling each whole bale was individually ground through a FitzMill (FitzMill Comminutor, Fitzpatrick, Elmhurst, IL) with a screen size of 4.76 mm. The shredded biomass was collected and mixed thoroughly for two minutes using a twin shaft paddle mixer (Hayes & Stolz, Fort Worth, TX). From the homogenized mixture, sub-samples were collected for compositional analysis (about 1 kg) and for further particle size reduction through a Bliss Hammer Mill (Bliss Industries, Ponca City, OK) with screen size of 0.397 mm attached to a Craftsman ShopVac (Sears Holdings Corp., Hoffman Estates, IL). Samples

ground through the Bliss Mill were collected, mixed and further sub-sampled for conversion to ethanol, moisture content determination and measurement of extra-cellular enzymatic activity.

2.4 Biomass Component Determination

Biomass components were determined by the Ruminant Nutrition Lab of the Department of Animal Sciences at Kansas State University, Manhattan, KS. Sub-samples from three bales from each treatment group at each sampling time were collected after bale grinding and immediately used for biomass component analyses. Analyses included dry matter determination using AOAC Method 930.15, neutral detergent fiber (NDF) using ANKOM Method 6, acid detergent fiber (ADF) using ANKOM Method 8 and acid detergent lignin (ADL) using ANKOM Method 8 (ANKOM Technology, USA). ADF, NDF and ADL values were used to obtain cellulose, hemicellulose and lignin content of the baled biomass sub-samples. In addition, moisture content of each bale at the sampling time was determined using AACC International Method 44-19.01

2.5 Extra-Cellular Enzyme Activity Assay

Cellulase and hemicellulase activities were determined using a fluorimetric, 96-well plate assay based on the method outlined by Marx et al. (2001), listed in table 1 (all substrate and buffers from Sigma-Aldrich, St. Louis, MO). In brief, a 1 g sample of each ground bale was mixed with 100 ml of 50 mM sodium acetate pH 7.0 and sonicated for 2 min to release any enzymes from the ground samples. From the sonicated sample, 20 µl was loaded into designated substrate and standard curve wells of the 96-well plate. Plates were loaded with four substrates linked to 4-methylumbelliferone for determination of enzymatic activity based on fluorescence. The linked substrates were loaded into the plate at final substrate concentrations of 200 and 400 µM, both in duplicate. The standard curve was constructed using 4-methylumbelliferone at a final concentration of 0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0 and 50 µM. 50 mM sodium acetate pH 7.0 was loaded into the plate first, followed by 4-MUB for the standard curves,

4-MUB linked substrates and then samples. Plates were incubated at 30°C for two hours. After incubation, 10 µl of 1 M sodium hydroxide was added to each well to increase the pH beyond 10 to maximize MUB fluorescence. Fluorescence was measured by a computerized microplate fluorimeter (Victor 3, PerkinElmer, Waltham, MA) with 355 nm excitation and 460 nm emission filters. The enzymatic activity was estimated by regression based on the standard curves.

2.6 Conversion to Ethanol

As with the biomass component determination, aliquots from three bales from each treatment and sampling time were used for conversion to ethanol. The conversion of sorghum biomass to ethanol followed methods outlined by Yoo et al (2011) with some modifications. In brief, substrate (20% w/w) was added to 2% (w/w) sodium hydroxide solution (Sigma Aldrich, St. Louis, MO) and autoclaved (SS-325E; Tomy Tech, USA, Inc., Fremont, CA) at a temperature of 121°C for 30 minutes. The remaining solids were washed with deionized water to neutralize, followed by drying in an air oven at 60°C for 24 h. Enzymatic hydrolysis of the dried pretreated substrate was carried out using the proprietary blend of cellulase enzymes, Cellic® CTec2, provided by Novozymes (Novozymes A/S, Denmark). Enzymatic hydrolysis was carried out with 3.0% enzyme per total solids (g enzyme/g biomass) in 0.1 M citric acid buffer pH 5.0 at 50°C for 48 h and shaking at 120 RPM. The hydrolyzed substrate was inoculated at2% (v/v) with actively growing *Saccharomyces cerevisiae* Ferm Pro™ (Danville, KY) and incubated at 30°C for 24 h with shaking at 100 RPM. In addition, 3% (v/v) of a 10% yeast extract solution (BD Biosciences, San Jose, CA) was added at inoculation of the substrate. Actively growing *S. cerevisiae* was obtained through propagation of -80°C glycerol stock cultures in yeast mold broth (BD Biosciences, San Jose, CA) for 24 h at 30°C. 1 mL samples were taken after enzymatic hydrolysis and fermentation for quantification of glucose and ethanol using a binary HPLC system (Shimadzu Corporation, Japan) as described by Oberoi et al (2011) and a Phenomenex Rezex RPM monosaccharide column (300 x 7.8 mm; Phenomenex, CA).

In brief, degassed deionized water was used as a mobile phase at a flow rate of 0.6 mL min^{-1}. The column oven and refractive index detector (RID-10A) were maintained at 80°C and 65°C, respectively. Samples were centrifuged and filtered through Phenomenex 0.45 micron RC membranes prior to injection. Peaks were detected by the refractive index detector and quantified on the basis of area and retention time of the standards (glucose and ethanol).

2.7 Data Analysis

The data were analyzed using PROC GLIMMIX in SAS 9.2 Software (Cary, North Carolina, USA). The treatment and time main effects and treatment*time interactions were tested, followed by post-hoc analysis of treatment. The effects of storage time were evaluated against treatment NN at time 0 as the control. The treatment*time interactions were further decayed with pairwise comparisons of slice effects. Differences were considered significant at alpha = 0.05. Statistical differences between treatments at a sampling point are indicated by different letters, while * indicates a statistical difference from the control (treatment NN at time 0).

3. Results and discussion

This study provides detailed information on biomass components, extra-cellular enzyme activity and ethanol yields in dry, baled biomass stored under the four storage conditions over a 6 month period. All dry weights are based on the post-storage moisture content of each bale. At sampling time 0, the average moisture content was determined to be 10-12%, which was also assumed to be the initial moisture content of all the bales. The bales in the uncovered treatment were visibly deteriorated (dark colored, moldy), whereas those from the covered treatments were similar to their initial condition after baling, with little to no noticeable deterioration, even after 6 month storage.

3.1 Biomass Component Determination

Sorghum biomass dry matter, cellulose and hemicellulose contents were generally similar in treatment NT, PN and PT, but differed from treatment NN after storage (Figure 1), while lignin content was similar between all treatments and storage times, including NN. We focus on the differences between the three covered treatments (NT, PN and PT) and the uncovered, control treatment (NN) at each sampling time. As expected, biomass components did not differ among the treatments at time zero. Dry matter did not change after 6 months of storage in the covered treatments, shown in figure 1a. In contrast, dry matter content declined in treatment NN, which also differed from treatments NT, PN and PT after 2, 4 and 6 month storage. Our dry matter results concur with Khanchi et al. (2009), who baled forage sorghum into large square or round bales and stored them either without cover or covered with a tarp. Khanchi et al. (2009) reported that the dry matter losses in uncovered bales stored outdoors ranged from 5.73 to 6.04% after 6 months of storage, whereas dry matter losses in the tarp-covered bales ranged from 5.73 to 6.34%. Shah et al. (2011) reported dry matter losses of 11% in corn stover bales covered with a tarp and 17% dry matter losses in bales wrapped in plastic after 9-month storage. Dry matter losses were more dramatic in this study: uncovered small square bales lost on average 33.2% of dry matter during the 6-month storage. These differences among the studies are likely attributable to bale geometry, bale size, bale density, biomass feedstock, and sampling methods.

Cellulose contents in the covered treatments NT, PN and PT remained stable compared to the uncovered treatment NN after 2, 4 and 6 month storage (figure 1b). In contrast to dry matter, cellulose contents in all treatments after 4 month storage were lower than in the control, with treatment NN having the lowest content of 22.4%. This continued decline in cellulose content for treatments NT, PN and PT was not seen after 6 months storage. While cellulose content in treatments NT and PT did not differ from the control after 6 month storage, it was lower in treatment PN than in the control (figure 1b). The reduced cellulose content in treatments NT, PN and PT after 4 months storage could be attributed to environmental conditions, as sampling occurred in mid-February when temperatures and

precipitation were at a low (figure 2). Additional environmental factors that could have led to biomass degradation are temperature cycling and extremes, moisture (precipitation) and solar degradation. However, at the 6 months storage time, reduced cellulose content was not seen in treatments NT, PN and PT compared to time 0. This could indicate that reduced temperatures and precipitation (seen after 4 months of storage) could greatly influence cellulose degradation, even when the biomass is covered.

The change in hemicellulose content was found to follow the same pattern as dry matter. The uncovered treatment NN was found to have reduced hemicellulose content after 2, 4 and 6 month storage, while the content in the covered treatments (NT, PN and PT) remained stable (figure 1c.). Lignin contents did not differ among the treatments at sampling point (figure 1d). However, treatment NN differed from control after 2, 4 and 6 months storage. As seen with cellulose and hemicellulose contents, a decrease in lignin content was observed after 4 months storage for all treatments, which could be due to environmental factors at sampling as previously described. Our observations are consistent with those of Shah et al. (2011), who also reported stable cellulose, hemicellulose and lignin contents after 9 months storage of large square corn stover bales covered with a tarp or wrapped in plastic.

3.2 Extra-cellular Enzymatic Activity (EEA) Assay

The MUB-linked substrates allowed for the assessment of cellulose and hemicellulose degrading extra-cellular enzyme activities in plant biomass used for ethanol production and stored for 0, 2, 4 and 6 months under four conditions. Such fluorometrically linked substrates have been primarily used for estimation of extra-cellular enzyme activities in a wide variety of soil and litter samples (DeForest, 2009; Saiya-Cork et al., 2002; Selmants et al., 2005), but not specifically for plant biomass samples. Enzymatic activities reported here are similar or slightly higher than those in soil and litter samples with comparable moisture contents (DeForest, 2009; Selmants et al., 2005), as might be expected based on

the abundance of (hemi-)cellulose in our biomass. Cellulose (4-MUB- β-D-cellobioside and 4-MUB- β-D-glucoside) and hemicellulose (4-MUB- β-D-glucuronide hydrate and 4-MUB-β-D-xyloside) degrading enzyme activities are shown in figure 3. As with the biomass components, treatment NN at time 0 was considered as a control and none of the EEAs differed among the treatments at time 0. Cellulose degrading activities in the covered treatments NT, PN and PT did not differ across storage times and did not differ from the control after any length of storage (Figures 3a and 3b). In contrast, these activities were higher in the uncovered treatment than in the control after 2, 4 and 6 month storage. Although the two substrates used to estimate the activities of cellulose-degrading enzymes were largely consistent, cellulase activity as measured by 4-MUB-β-D-glucoside was higher in treatment PT than in the control and did not differ from treatment NN after 6 month storage. Hemicellulase activity as measured by 4-MUB- β-D-glucuronide hydrate (figure 3c) did not differ between treatments or when compared to the control after any length of storage, except for treatment PT where estimated activity was higher than in the control after 6 month storage. Hemicellulase activity as measured by 4-MUB-β-D-xyloside did not differ between treatments at time zero as expected. The estimated activity in treatment NN was greater than in the control after 2, 4 and 6 months of storage. Furthermore, this activity was higher in treatment NN than in treatments NT, PN and PT after 2, 4 and 6 month storage. Treatments NT, PN and PT did not differ at 2 and 4 month storage, but differed between treatments after 6 months. Overall, the cellulose and hemicellulose degrading extra-cellular enzyme activities were consistently highest in the uncovered bales. This corroborates with the reduced cellulose and hemicellulose contents in these bales. In contrast, the enzyme activities remained low in the covered bales and the cellulose and hemicellulose contents stable after 6 months of storage.

3.3 Conversion to Ethanol

Prior to enzymatic hydrolysis, we optimized sample pretreatment using a dilute acid (2% sulfuric acid) or dilute alkali solutions and autoclaving (results not shown) as described in Brijwani et al (2010) and Oberoi et al (2011). The dilute alkali pretreatment (protocol described above) resulted in the highest glucose yield from the biomass and was therefore chosen for further evaluation for the production of ethanol. We also evaluated optimal parameters for enzymatic hydrolysis and fermentation to ethanol by varying enzyme dosage levels and incubation times (results not shown). Figures 4a and 4b display glucose and ethanol yield per gram of biomass on dry weight basis (prior to pretreatment) displayed. As with the other parameters (biomass components and extra-cellular enzymatic activity) we determined, the glucose and ethanol yields did not differ among the treatments at time 0. We observed no differences in glucose yields between the treatments or between treatments and the control after up to six months of storage, except for an increase in the uncovered treatment NN after 2-month storage compared to the control. Our glucose yields were higher than those reported by Brijwani et al. (2010) and Yoo et al. (2011) for enzymatic hydrolysis of soybean hulls and soybean hulls supplemented with wheat bran, respectively. These differences can be attributed to differences in feedstock component quantities and in the chosen enzyme hydrolysis system. Similarly to glucose yields, ethanol yields did not differ between the treatments at time zero. None of the treatments differed from each other or from the control after 2 or 4 months storage in regards to ethanol yields. However, after 6 months, ethanol yields from biomass in treatment NN were lower than those from treatments NT, PN and PT and from the control. Ethanol yields in treatments NT, PN and PT did not differ from each other or from the control after six months storage. Ethanol yields after 0, 2 and 4 months of storage are similar to those reported by Balat (2011) for conversion of cornstalk to ethanol using dilute alkali pretreatment and enzymatic hydrolysis. During 6-month storage, ethanol yield from covered bales remained stable. In contrast, uncovered bales had a significant decrease in ethanol yields after 6 months of storage. Although the uncovered biomass contained less cellulose, we did not observe congruent decreases in

the glucose yields. This may indicate that biomass storage either uncovered or covered does not compromise the ability of the commercial enzymes to hydrolyze cellulose to glucose during enzymatic hydrolysis. Furthermore, the reduced ethanol yields from biomass stored uncovered for 6 months, observed in this study, could indicate the introduction of compounds inhibitory to ethanol fermentation by *S. cerevisiae*. In addition, it is important note the large loss of dry matter from the biomass stored uncovered - these losses will ultimately reduce the ethanol volume produced from biomass stored uncovered.

Substrate losses, including dry matter, cellulose and hemicellulose, were consistently greatest in uncovered stored plant biomass left susceptible to environmental elements. The results of this study strongly indicate plant biomass substrate used for the production of lignocellulosic ethanol can be preserved during storage if the biomass is covered.

4. Conclusions

From biomass harvest to conversion to ethanol, the storage method (covered or uncovered) for preserving dry plant biomass used for lignocellulosic ethanol production was found to play a large role in substrate quality and subsequent ethanol yields. This comprehensive study found congruency in three datasets, specifically reduced cellulose content, increased extra-cellular enzymatic activity associated with cellulose degradation and ultimately reduced conversion to ethanol yields in biomass left uncovered over six month storage. Congruency was also seen in covered biomass bales, with stability in cellulose content, extra-cellular enzymatic activity and ethanol yields.

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Tables and Figures

Table 1

Extra-cellular enzymes assayed in biomass samples collected from each treatment at each sampling point, their commission number (EC) and corresponding substrate.

Enzyme	EC	Substrate
Cellulose Degrading		
Cellobiohydrolase	3.2.1.91	4-MUB-β-D-cellobioside
β-1,4-Glucosidase	3.2.1.21	4-MUB-β-D-glucoside
Hemicellulose Degrading		
β-glucuronidase	3.2.1.31	4-MUB-β-D-glucuronide hydrate
β-1,4-Xylosidase	3.2.1.37	4-MUB-β-D-xyloside

4-MUB=4-methylumbelliferyl

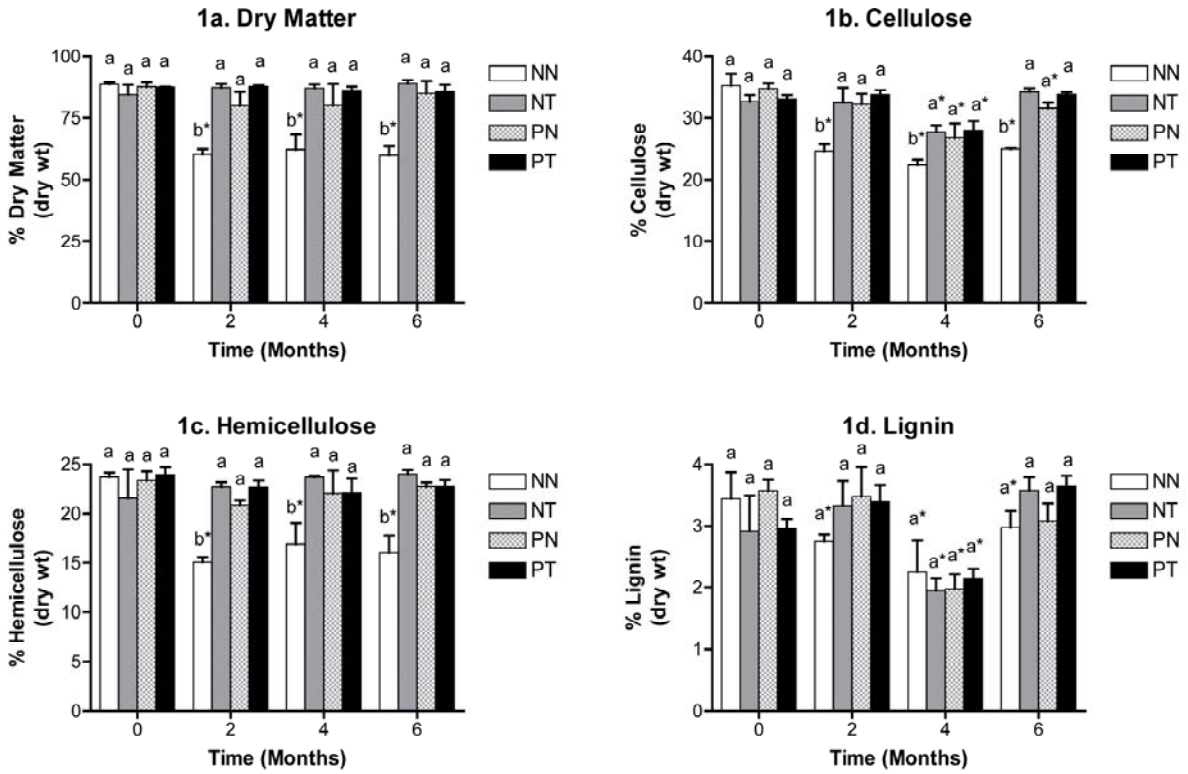


Fig. 1 –Changes in biomass components, including dry matter, cellulose, hemicellulose and lignin as percentage on dry basis. Different letters indicate significant differences within storage period, while * indicates significant difference from the control (treatment NN at time 0).

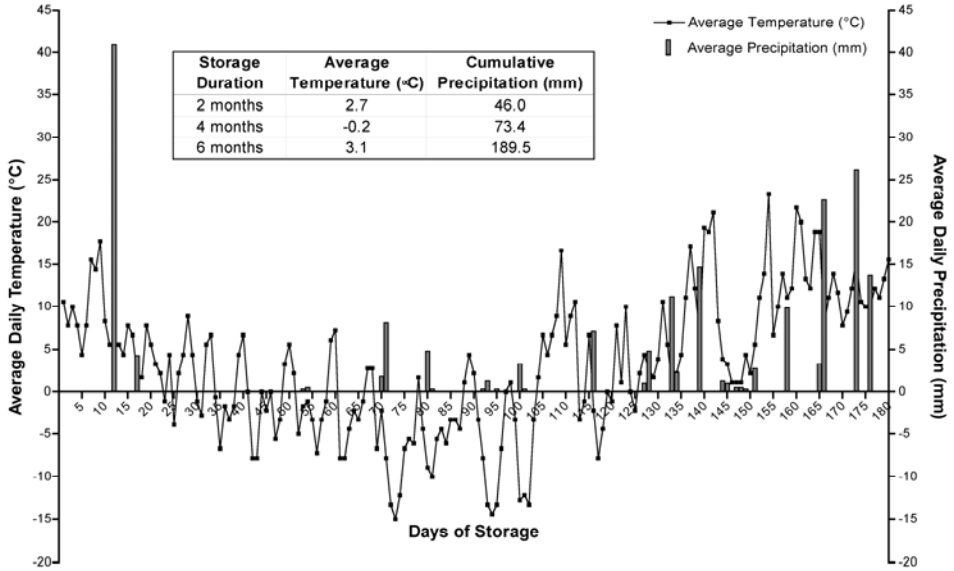


Fig. 2 – Daily temperature and precipitation averages during plant biomass stored under 4 different conditions (NN, NT, PN and PT) and 4 storage durations (0, 2, 4 and 6 months) along with average temperatures and cumulative precipitation for each storage time. 2 months storage occurred after 60 days on December 13th, 4 months after 120 days on February 21st and 6 months after 180 days on April 18th.

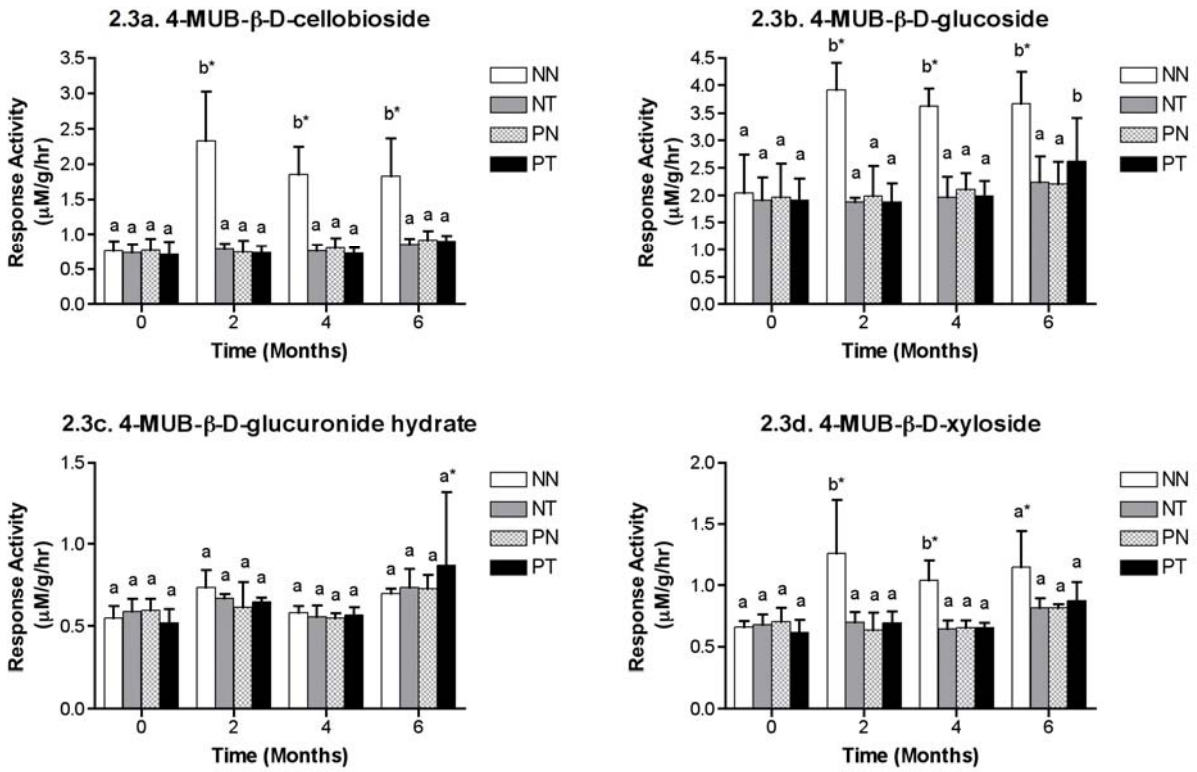
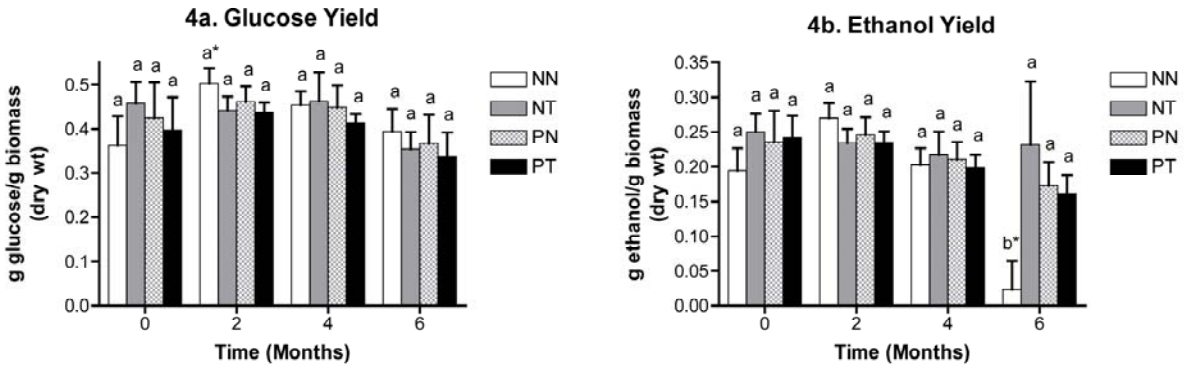


Fig. 3 – Cellulose degrading enzyme activity as measured by 4-MUB-β-D-cellobioside (3a) and 4-MUB-β-D-glucoside (3b) and hemicellulose degrading enzyme activity as measured by 4-MUB-β-D-glucuronide hydrate (3c) and 4-MUB-β-D-xyloside (3d) for all treatments over six months of storage. Activities are given in μM of activity per g of biomass per hour on dry weight basis. Different letters indicate significant differences within sampling time, while * indicates significant difference from the control (treatment NN at time 0).



1

2 Fig. 4 – Glucose (4a) and ethanol yields (4b) across treatments over time. Values given on dry weight
3 basis. Different letters indicate significant differences within sampling time, while * indicates significant
4 difference from the control (treatment NN at time 0).