

High-solids enrichment of thermophilic microbial communities and their enzymes on bioenergy feedstocks

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Abstract

Thermophilic microbial communities that are active in a high-solids environment offer great potential for the discovery of industrially relevant enzymes that deconstruct bioenergy feedstocks. In this study finished green waste compost was used as an inoculum source to enrich microbial communities and associated enzymes that hydrolyze cellulose and hemicellulose during thermophilic high-solids fermentation of switchgrass and corn stover. Methods involving the disruption of enzyme and plant cell wall polysaccharide interactions were developed to recover xylanase and endoglucanase activity from deconstructed solids. Xylanase and endoglucanase activity increased by more than a factor of 5, upon four successive enrichments on switchgrass. Overall, the changes for switchgrass were more pronounced than for corn stover; solids reduction between the first and second enrichments increased 4-fold for switchgrass while solids reduction remained relatively constant for corn stover. Pyrosequencing analysis of enriched samples indicated rapid changes in the microbial community between the first and second enrichment with the simplified community achieved by the third enrichment. The results demonstrate a successful approach for enrichment of microbial communities and enzymes active in a thermophilic high-solids environment.

Introduction

Plant residues and dedicated herbaceous crops have been proposed as viable sources of biomass for liquid fuel production. Limiting factors to their utilization include sustainable harvest, transportation, storage and conversion to fermentable sugars. Recalcitrance to conversion has been identified as one of the most challenging factors facing utilization (Bohlmann 2006; Chandra et al. 2007; Yang and Wyman 2008). This is due in part to expensive deconstruction and hydrolysis of plant cell wall polymers. Industrially robust processes for the conversion of plant cell walls (lignocellulose) to liquid fuels or other products will require enzymes that function under extremes in temperature, solute concentrations and moisture (Kulkarni et al. 1999; Rubin 2008). Sources of such enzymes include communities that are currently subjected to many of the extreme conditions expected in industrial plant cell wall conversion systems. One such community is from composting which involves the controlled aerobic decomposition of organic matter (VanderGheynst et al. 1997). Composting processes typically include a thermophilic phase to inactivate human and plant pathogens and weed seeds. Many processes are tuned to facilitate introduction of air and moisture (Marshall et al. 2004). The result is a microbial community that is remarkably robust with the metabolic potential to rapidly degrade plant cell wall polysaccharides in a thermophilic, high-solids environment (Allgaier et al. 2010). These communities offer great potential for the discovery of enzymes that deconstruct plant cell walls.

In addition to process engineering challenges related to feedstock acquisition and conversion, water will likely be an expensive resource and residue stream to be managed in biological systems employing plant biomass conversion to fuels (Gerbens-Leenes et al. 2009). Operating hydrolysis and fermentation processes at low moisture levels (high solids) reduces costs associated with water purchase and treatment. High solids conversion processes provide other advantages for industrial scale conversion. For example, lower water levels translate to a lower heat capacity of liquid-biomass mixtures reducing energy requirements for heating and cooling. Costs for product recovery also decrease with decreasing water in the product stream. However, high solids environments present challenges associated with mixing, and more rapid feedback inhibition from hydrolysis products (Merino and Cherry 2007; Zhang et al. 2010). Identification of enzymes that remain active under such conditions would be valuable for industrial scale processes.

The goals of this study were to demonstrate an approach for selecting microbial communities and secreted enzymes that decompose bioenergy feedstocks in a thermophilic high-solids environment, and to examine the evolution of microbial community structure and enzyme activity during enrichment. Green waste compost was used as a source of inoculum, and switchgrass and corn stover were the sources of feedstock. Both feedstocks show potential for bioenergy production and reducing greenhouse gas emissions (Schmer et al. 2008; Spatari et al. 2005).

To facilitate the measurement of enzyme activity, experiments were necessary to identify extraction conditions that recovered enzymes bound to or trapped within the plant cell wall matrices. Extraction buffer compounds selected for investigation included Tween 80, NaCl and ethylene glycol. Recognizing that the composition of corn stover (cobs, leaves and stems) and switchgrass (leaves and stems) are very different, two separate studies were completed to identify extraction conditions for each feedstock.

Microbial community structure was monitored using small-subunit ribosomal RNA (SSU rRNA) amplicon pyrosequencing based on the high-throughput Roche 454 sequencing platform. Microbial diversity was determined after each incubation cycle over the course of the entire experiment to follow temporal changes and to determine adaptation of specific phylotypes to the provided feedstocks.

The results from the study demonstrate a successful high-solids approach for selecting microbial communities and secreted enzymes for biomass deconstruction. Disruption of hydrophobic interactions between enzymes and plant cell wall polysaccharides was imperative for yielding xylanase and endoglucanase activities from enriched samples.

Materials and Methods

High-solids incubations

Finished green waste compost was obtained from a commercial facility that composts agricultural residues including tree and vine prunings (Grover Soil Solutions, Zamora, CA). Compost was solar-dried and stored at 4°C until applied as inocula.

Corn stover was obtained courtesy of Bruce Dale of Michigan State University (MSU), East Lansing, MI. Corn stover with corn cobs without grains (NK brand N33-J4) were harvested

from MSU corn fields in 2008. Switchgrass stem pieces were obtained from the laboratory of Dr. Ken Vogel of USDA-ARS, University of Nebraska, Lincoln, NE.

The biomass was size reduced using a leaf shredder and air dried until the moisture was less than 10%. Then it was further size reduced using a Wiley mill with a 10 mm screen. Both feedstocks were extracted with ethanol for 1.5 days and water for 2 days in a Soxhlet extractor. The materials were then dried in a vacuum oven for 4 days and stored in air-tight containers at 4°C until needed.

Prior to incubation, extracted switchgrass and corn stover were wet with minimal media to a target moisture content of 400 wt% dry basis ($\text{g water g dry solid}^{-1}$) (80 wt% wet basis ($\text{g water g total}^{-1}$)) and allowed to equilibrate at 4°C overnight. Feedstocks were inoculated with 10 wt% ($\text{g dry compost g dry solid}^{-1}$) compost immediately before incubation.

High-solids incubations were done for each feedstock and conducted as described previously (Reddy et al. 2009). Briefly, bioreactors with a 0.2 L working volume were loaded with 10 – 11 g dry weight of feedstock and compost mixture. Air was supplied to each bioreactor at 10 mL/min to maintain aerobic conditions. Incubator temperature was maintained at 35°C for 1 day, ramped to 55°C over one day, and held at 55°C for the duration of the experiment. Temperature was monitored with a HOBO data logger (Onset Computer Corporation, Bourne, MA). To maintain a moisture content ideal for microbial activity, water was added to each bioreactor and the contents were mixed every 3.5 days.

The respiration rate of the microbial community, represented as CO₂ evolution rate (CER), was measured for all incubated samples. Carbon dioxide concentration was measured on the influent and effluent air of the bioreactors using an infrared CO₂ sensor (Vaisala, Woburn, MA) and flow was measured with a thermal mass flow meter (Aalborg, Orangeburg, NY). Carbon dioxide and flow data were recorded every 20 min using a data acquisition system; carbon dioxide evolution rate (CER) and cumulative respiration (cCER) were calculated as described previously (Reddy et al. 2009).

For experiments to identify the enzyme extraction buffer, one bioreactor for each feedstock was inoculated and aerated for eight days before harvesting. For the long-term enrichment study, each feedstock was incubated in triplicate; three switchgrass and three corn stover communities were allowed to develop in parallel. Every two weeks, feedstock samples containing each enriched community were collected. Fresh feedstock was inoculated with 10

wt% (g dry enriched sample g total dry weight⁻¹) of the enriched community and transferred to a new bioreactor. The long-term enrichment experiment ran for eight weeks yielding a total of four time points (T1, T2, T3 and T4) for enzyme and microbial community analyses.

At the end of each incubation samples were collected for measurement of enzyme activity, microbial community structure (long-term incubation only) and moisture content. Methods for enzyme activity and microbial community structure are described in detail in later sections of this article. Moisture content was measured gravimetrically after drying samples at 105°C for 24 h.

Identification of buffer components for enzyme extraction from solid samples

Components for the enzyme extraction buffer were identified using full factorial studies with three center-point replicates (Table 1). Each study was conducted with the three following components: ethylene glycol (0 – 50 wt%), Tween 80 (0.01 – 0.15 wt %) and sodium chloride (0.1 – 1.5 wt %). A sodium acetate buffer (50 mM, pH = 5.0) control was also examined.

Three grams (wet weight) of freshly harvested colonized feedstock was shaken with 27 g of each buffer for 60 minutes at 150 RPM and room temperature. Samples were centrifuged at 4°C and 10,000 x g for 20 min and then filtered using 0.2 µm membranes.

To reduce interference of the buffer components with enzyme activity measurements, the extraction buffer was exchanged with sodium acetate buffer before proceeding with the enzyme activity assays. VivaSpin columns with a PES membrane and a 5 kDa molecular weight cut off (VWR, West Chester, PA) were used.

Activity in dialyzed extracts was measured as described below. JMP statistical software (v. 8.0.1, SAS Institute, Cary, NC) was used to perform stepwise regression and determine significant buffer components. Data were used to determine the ideal component levels for the extraction buffer for each feedstock.

Enzyme extraction from enriched samples and activity assays

Proteins were extracted from incubated switchgrass and corn stover samples according to the procedure described previously using 50 wt% ethylene glycol, 0.1 wt% Tween 80 and 1 wt% sodium chloride.

Enzyme activities were measured using a 96-well plate reducing sugar assay. All incubations were conducted in an Eppendorf Mastercycler PCR thermal cycler with heated lid (Eppendorf North America, Hauppauge, NY). Cellulase was measured as described by Xiao et al. (Xiao et al. 2005) with the following modifications: the total working volume in each well was 160 μ L, the 50 mM sodium acetate buffer had a pH of 5.0, and the incubation times were longer. The substrate was 2% carboxymethylcellulose (CMC) and the standard curve was made with sterile-filtered glucose dilutions.

Briefly, 40 μ L of sample was incubated with 40 μ L of substrate at 50°C, to allow conversion of cellulose to sugars. The reaction was stopped with 80 μ L of a dinitrosalicylic acid (DNS) reagent containing 1.4% DNS, 1.4% sodium hydroxide, 28% Rochelle salts, 0.28% phenol and 0.07% sodium sulfite. Sample blanks and standards were added, and the plate was incubated at 95°C for 5 min to develop color proportional to the concentration of sugar in the well. Samples were transferred to round-bottom spectrophotometer plates and the color was measured at 540 nm.

To measure xylanase activity, the method described above was combined with the protocol developed by Bailey et al. (Bailey et al. 1992; Xiao et al. 2005). The xylanase protocol was identical to the 96-well cellulase assay described above, but the substrate was 1% birchwood xylan, the standard curve was made with sterile-filtered xylose dilutions, and incubation times were shorter.

DNA extraction & SSU rRNA amplicon pyrosequencing

Samples from bioreactors at each time point were snap frozen in liquid nitrogen and homogenized with an oscillating ball mill (MM400, Retsch Inc., Newtown, PA). DNA was extracted with a CTAB and bead-beating protocol described elsewhere (Allgaier et al. 2010). A ~450 bp fragment of the small-subunit (SSU) rRNA gene was amplified using the broadly conserved primer pair 926F-1392R as described in Kunin et al. (Kunin et al. 2010). The reverse primer included a 5 bp barcode for multiplexing of samples during sequencing. Barcoded amplicons were mixed in equal proportions prior emulsion PCR following manufacturer's instructions and sequenced using the Roche 454 GS FLX Titanium technology. Sequencing tags were quality trimmed and analyzed using the pyroclust version of the software tool PyroTagger

(<http://pyrotagger.jgi-psf.org>) with a 220 bp sequence length threshold and an accuracy of 10 % for low quality bases.

To reduce noise in statistical analysis all singleton OTUs were removed from the data set. Non-metric multidimensional scaling (NMDS) analyses and calculation of Shannon's diversity indices were performed using the vegan package in the R software environment (<http://CRAN.R-project.org/package=vegan>). NMDS parameters were set to 3 dimensions and a maximum of 1000 random starts. Similarity percentage (SIMPER) analysis were performed using the respective function in the PRIMER V software package (Plymouth Marine Laboratory, Plymouth, UK) using default settings.

Results

The ideal extraction buffer for enzyme recovery from incubated solids was determined from a full factorial study in which incubated samples of corn stover and switchgrass were extracted using the components and levels listed in Table 1. The largest variations in enzyme activity extracted were for xylanase (Table 1). Xylanase activities from switchgrass varied between 0.29 IU g dw⁻¹ for sodium acetate extraction to 0.71 IU g dw⁻¹ for extractions containing 50 wt% ethylene glycol and 0.15 wt% Tween 80. Similar to switchgrass, xylanase extraction from corn stover was also highest (0.37 IU g dw⁻¹) when extractions contained 50 wt% ethylene glycol.

Endoglucanase extraction also varied with the composition of the buffer, but differences were much smaller compared to xylanase. Like xylanase, the greatest activities were observed with extractions containing 50 wt% ethylene glycol. These were 0.18 IU g dw⁻¹ and 0.13 IU g dw⁻¹ for switchgrass and corn stover, respectively.

For xylanase and endoglucanase extractions from switchgrass, sodium chloride and Tween 80 had no significant effect (p-values > 0.05) on enzyme activity. However, ethylene glycol had a significant positive effect on the extraction of xylanase activity (p-value = 0.0002) and endoglucanase activity (p-value = 0.0071).

For corn stover, ethylene glycol had a significant positive effect on xylanase activity extracted (p-value = 0.0002). Also, the interaction between Tween 80 and ethylene glycol was significant; when ethylene glycol was at 50 wt% in the buffer, increasing Tween 80 from 0.01 wt% to 0.15 wt% had a negative effect on xylanase activity (p-value = 0.0168). Sodium chloride had no significant effect on xylanase activity (p-value > 0.05).

Ethylene glycol also had a significant positive effect on endoglucanase activity extracted from corn stover (p-value = 0.0056), while sodium chloride had a significant negative effect (p-value = 0.0036). Also, the interaction between sodium chloride and Tween 80 had a significant negative effect (p-value = 0.0368); when NaCl was present at a 1.5 wt% in the buffer, increasing Tween 80 from 0.01 wt% to 0.15 wt% had a negative effect on endoglucanase extracted.

The conditions selected for extracting enzymes from enriched samples were based on results from the screening study and the ease of sample handling. Extractions with higher than 50 wt% ethylene glycol had high viscosity and were difficult to mix. For this reason, the maximum ethylene glycol level was set at 50 wt%. Tween 80 and sodium chloride were also included at moderate levels of 0.1 wt% and 1 wt%, respectively, because their presence was found to reduce enzyme adsorption to plastic vials used in sample processing and assays (data not shown).

Microbial activity during successive enrichments of switchgrass and corn stover

For all enrichment incubations with a particular feedstock, replicate treatments had similar respiration profiles (Figure 1). Respiration profiles for the first pass (T1) are shown in Figure 1 a and c for switchgrass and corn stover, respectively. During the first two-week incubation, respiration rate peaked around 1.5 days and dropped significantly for both feedstocks. At day 11, a second peak occurred for switchgrass, indicating adaptation of the community to decompose recalcitrant compounds under thermophilic conditions.

Successive respiration profiles showed a more sustained period of activity after the transfer and then a steady decline. This trend was consistent for all time points after T1. Respiration profiles for the last enrichment of the study (T4) are shown in Figure 1 b and d for switchgrass and corn stover, respectively. Microbial activity on both feedstocks peaked in less than one day and steadily declined. Peak respiration rates varied between 40-50 mg CO₂ day⁻¹ g dw⁻¹ for both feedstocks; however, corn stover respiration rates declined more rapidly compared to switchgrass. This led to differences in cumulative respiration between feedstocks.

In general, total microbial activity, represented as cumulative carbon dioxide evolution (cCER), was greater on switchgrass compared to corn stover (Table 2). cCER increased with each transfer for switchgrass. For corn stover, cCER was greatest after the second two-week incubation (T2) and remained relatively constant with successful enrichments. After each two-

week incubation period, adapted communities reduced solids an average of 30% for switchgrass and 22% for corn stover (Table 2). Overall, the changes for switchgrass were more pronounced than for corn stover; solids reduction between the first and second time points increased 4-fold for switchgrass while reduction remained relatively constant for corn stover.

Enzyme activity

Enzymes were extracted from feedstocks with the selected buffer containing 1 wt% sodium chloride, 0.1 wt% Tween 80 and 50 wt% ethylene glycol. Enzyme activity increased for each successive transfer of switchgrass. From T1 to T4, xylanase and endoglucanase activity increased more than 5-fold. This corresponds to the increase in cCER and increase in solids reduction with each successive transfer (Table 2).

Enzyme activities were lower for corn stover compared to switchgrass and showed less variation with time, corresponding to the relatively constant cCER and solids reduction with enrichment. With the exception of xylanase activity in R13, the most activity for corn stover enrichments was seen at T2, the time point with the highest cCER.

Microbial community structure and dynamics

Microbial community structure was determined for all samples after each two-week incubation (T1 to T4) using small-subunit (SSU) rRNA gene amplicon pyrosequencing. All enrichment cultures indicated a wealth of phylogenetic diversity including bacteria and fungi, which decreased over time independent of the substrates used (Table 3).

Temporal succession in microbial diversity was also reflected in overall community composition as indicated by NMDS comparisons (Figure 3). The most dramatic shift in community composition occurred after T1 for both the switchgrass and corn stover communities. After T2 the communities stabilized and changes were only of minor significance. However, in the corn stover enrichments, the T2 samples indicate an intermediate stage between the T1 and the T3 and T4 samples by forming its own discrete cluster (Figure 3).

As summarized in Tables 4 and 5, some microorganisms were heavily enriched on the provided substrates whereas others completely disappeared. For example, a member of the genus *Microbispora* increased in relative abundance from initial 1% to over 30% at the later time

points. At the same time a member of the genus *Dactylosporangium* disappeared even though being dominant (> 10%) in the T1 samples.

To determine if the two substrates enriched for different communities, the T4 samples were compared using SIMPER (SIMilarity PERcentage) (Table 6). This method assesses which taxa are primarily responsible for differences between switchgrass and corn stover enriched communities. The four organisms belonging to *Paenibacillus* (Cluster 3), *Chelatococcus* (Cluster 4), Bacteroidetes (Cluster 6) and Gammaproteobacteria (Cluster 5) contributed most to the observed differences between the two substrates. Whereas members of Cluster 3, 4 and 6 appear to preferentially grow on switchgrass, Cluster 5 appears to be unique to corn stover.

Discussion

Switchgrass and corn stover were incubated in a high solids environment at 55°C with continuous aeration to simulate a composting process. Prior to initiating enrichment incubations, experiments were completed to identify extraction compounds required for recovery of enzymes from each feedstock. Three compounds that yielded positive results for other high solids biological systems were examined. Tween 80, a surfactant that disrupts non-specific binding of enzymes to substrates, was selected based on its positive effect on the desorption and recycling of cellulases during saccharification of crystalline cellulose and pretreated pine (Otter et al. 1989; Tu et al. 2007). Sodium chloride was investigated to increase the ionic strength of the buffer and disrupt interactions between protonated groups on proteins and feedstocks. It assisted in the recovery of xylanase from sugarcane bagasse (Maciel et al. 2009). The third component was ethylene glycol, which was selected to disrupt hydrophobic interactions. It also had a positive effect on the recovery of enzymes during recycling (Otter et al. 1989; Tu et al. 2007).

Ethylene glycol had the most significant effect on the extraction of enzymes from both switchgrass and corn stover. For xylanase, extracted activity doubled when ethylene glycol increased from 0 wt% to 50 wt% in the buffer. This implies strong hydrophobic interactions between the enzymes and substrate. The carbohydrate-binding modules (CBM) of well-studied cellulases have been shown to include polar residues that would facilitate hydrophobic interactions between proteins and polysaccharides (Beckham et al. 2010). The fact that ethylene glycol had such a significant impact on extracted enzyme activity suggests an enrichment of

enzymes with CBMs. These interactions would need to be disrupted for efficient recovery of enzymes decomposing solid substrates.

Long-term enrichments were carried out by incubating feedstocks and transferring the established community to fresh feedstock every two weeks. For switchgrass the increase in microbial activity, enzyme activities and solids reduction indicates continuing adaptation with enrichment. In contrast to switchgrass, there was a decline in microbial activity and enzyme activities after T2 enrichments with corn stover. The decline in activity suggests that compounds inhibitory to microbial and enzymatic activity accumulated during enrichments. A recent report indicated that extracts from corn stover (extracted using pH 7 buffer) included lignin-derived compounds that could be particularly inhibitory to microorganisms (Du et al. 2010). Possible inhibitory compounds include phenolic compounds, which are a byproduct of lignin degradation (Almeida et al. 2007; Palmqvist and Hahn-Hagerdal 2000). Lignin has been shown to decrease growth and enzyme activity in *Trichoderma reesei* (Vohra et al. 1980) and has been shown to inhibit cellulases and xylanases produced by thermophilic bacteria (Joh et al. 2010; Ximenes et al. 2010). To prevent inhibition in future adaptation studies, enriched samples could be leached prior to inoculating subsequent batches of feedstock. This approach was successful in prolonging growth and enzyme production by *Acidotherrnus cellulolyticus* during high-solids fermentation of switchgrass (Rezaei et al. 2010).

Pyrosequencing data showed simplification/specialization of the microbial community for both switchgrass and corn stover indicating selection of a few dominant community members adapted to decomposing these feedstocks in a high-solids, thermophilic environment. The dominant members of the adapted community (T4) on switchgrass were *Microbispora*, *Paenibacillus* and *Chelatococcus*, and on corn stover they were *Microbispora*, Gammaproteobacteria and Acidobacteria. Several members of the genus *Microbispora* have been isolated from high-solids environments and have demonstrated high levels of activity on xylan and other plant cell wall feedstocks (Hong et al. 2009). One approach for isolation of *Microbispora* from soil is treatment of samples under dry heat (Hayakawa et al. 1991). In addition, some species of *Microbispora* produce potent antibiotics (Foulston and Bibb 2010). The combination of antibiotic production and tolerance to dry conditions may have led to the dominance of *Microbispora* in both switchgrass and corn stover enrichments. Their presence may have also played a role in the microbial adaptation process.

Paenibacillus have been enriched in black liquor samples from paper pulping processes (Ko et al. 2007) and have been reported to secrete xylanase, cellulase and pectinase during enrichment on complex polysaccharides (Ko et al. 2007; Waeonukul et al. 2008). *Chelatococcus* have been isolated from hot springs and identified in mature compost piles (Panday and Das 2010; Székely et al. 2009) though have not been presented as a dominant community member in prior studies. To our knowledge, this is the first report of their significance in cell wall polysaccharide decomposition under thermophilic conditions. In contrast, it is not surprising to find Gammaproteobacteria and Acidobacteria on several of the enriched corn stover samples as they are commonly identified in thermophilic environments such as compost and are known to tolerate nutrient deprived and dry environments (Allgaier et al. 2010; Székely et al. 2009; Ulrich et al. 2008; Ward et al. 2009).

The corn stover and switchgrass in this study were inoculated with the same green waste compost source and incubated under identical conditions (temperature and aeration) simultaneously. The microbial community diverged by the first sampling point (T1) and resulted in different dominant members at all points of the enrichment study. Divergence of the community, even though both samples started with the same inocula, indicates plant biomass source has a tremendous impact on the types of organisms enriched. Enzyme activities, in particular xylanase, were much greater in switchgrass enrichments compared to corn stover. Such differences in the enriched community and enzyme activities demonstrate the need for specialized metabolic pathways and activities that depend on the unique plant cell wall polysaccharide composition of biofuel feedstocks.

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Table 1. Full factorial experimental design for buffer selection for enzyme extraction from switchgrass and corn stover and resulting enzyme activities in dialyzed extracts.

Coded Component Levels				Switchgrass		Corn stover	
Treatment	NaCl [*]	Tween 80 ^{**}	Ethylene Glycol ^{***}	Xylanase (IU g dw ⁻¹)	Endoglucanase (IU g dw ⁻¹)	Xylanase (IU g dw ⁻¹)	Endoglucanase (IU g dw ⁻¹)
1	0	0	0	0.31	0.11	0.22	0.10
2	+1	-1	-1	0.30	0.11	0.18	0.10
3	0	0	0	0.45	0.14	0.24	0.11
4	+1	+1	+1	0.71	0.15	0.31	0.10
5	-1	-1	-1	0.35	0.13	0.17	0.11
6	-1	+1	+1	0.71	0.18	0.32	0.13
7	+1	-1	+1	0.70	0.15	0.37	0.12
8	-1	-1	+1	0.59	0.17	0.33	0.13
9	+1	+1	-1	0.41	0.12	0.20	0.09
10	0	0	0	0.49	0.16	0.23	0.11
11	-1	+1	-1	0.36	0.14	0.21	0.12
12	Sodium Acetate buffer control			0.29	0.15	0.17	0.11

^{*} NaCl settings: -1 = 0.1 wt%, 0 = 0.8 wt%, +1 = 1.5 wt%

^{**} Tween 80 settings: -1 = 0.01 wt%, 0 = 0.08 wt%, +1 = 0.15 wt%

^{***} Ethylene Glycol settings: -1 = 0 wt%, 0 = 25 wt%, +1 = 50 wt%

Table 2. Cumulative carbon dioxide evolution rate (cCER) and average solids reduction measured after each two-week incubation period (T1–T4)

Sampling point	Switchgrass		Corn Stover	
	Average cCER (mg CO ₂ g dry feedstock ⁻¹)	Average reduction in total solids (wt%)	Average cCER (mg CO ₂ g dry feedstock IU g dw ⁻¹)	Average reduction in total solids (wt%)
T1	349	8	264	19
T2	424	29	355	22
T3	433	28	304	22
T4	466	34	288	23

Table 3. Temporal changes in microbial community on switchgrass (S) and corn stover (C) after each 2-week incubation (T1–T4): (a) Shannon diversity index and (b) richness (no. OTUs)

Reactor #	Feedstock	T1	T2	T3	T4
(a) Shannon Diversity Index					
S11	Switchgrass	3.77	2.15	2.36	2.14
S14	Switchgrass	3.67	2.47	2.53	2.40
S16	Switchgrass	3.96	2.70	2.76	2.47
C12	Corn stover	4.19	3.13	3.02	3.11
C13	Corn stover	3.92	3.80	3.06	2.93
C15	Corn stover	4.12	3.76	3.09	3.09
(b) Richness (no. OTUs)					
S11	Switchgrass	231	116	102	78
S14	Switchgrass	309	119	124	165
S16	Switchgrass	381	126	136	105
C12	Corn stover	397	185	161	163
C13	Corn stover	245	196	177	107
C15	Corn stover	387	224	159	141

Table 4. Changes in relative abundance of selected OTUs in the switchgrass (S) microbial community over time.

OTU	T1	T2	T3	T4	Taxonomy; greengenes ID (http://greengenes.lbl.gov)
S11					
Cluster1	0.9	32.8	36.6	35.1	Microbispora (Actinobacteria); 12149
Cluster3	2.1	37.1	17.7	15.5	Cohnella (Firmicutes); 27158
Cluster4	2.8	2.6	8.3	19.2	Chelatococcus (Proteobacteria); 232942
Cluster5	7.9	0.0	0.0	0.0	Sinobacteraceae (Gammaproteobacteria); 210648
Cluster11	10.5	0.1	0.1	0.2	Dactylosporangium (Actinobacteria); 336366
Cluster13	10.2	0.2	0.0	0.0	Sinobacteraceae (Gammaproteobacteria); 219056
Cluster51	9.8	1.8	0.2	0.0	Bacillales (Firmicutes); 14393
S14					
Cluster1	0.7	32.9	29.9	17.5	Microbispora (Actinobacteria); 12149
Cluster3	0.9	25.5	16.2	20.7	Cohnella (Firmicutes); 27158
Cluster4	3.4	2.4	9.4	10.3	Chelatococcus (Proteobacteria); 232942
Cluster11	20.6	0.2	0.0	0.0	Dactylosporangium (Actinobacteria); 336366
Cluster29	7.6	0.9	0.1	1.1	Thermobacillus (Firmicutes); 152793
S16					
Cluster1	0.5	30.1	25.4	34.4	Microbispora (Actinobacteria); 12149
Cluster3	1.2	24.6	16.2	11.1	Cohnella (Firmicutes); 27158
Cluster4	2.2	3.1	7.9	15.2	Chelatococcus (Proteobacteria); 232942
Cluster11	10.6	0.3	0.0	0.0	Dactylosporangium (Actinobacteria); 336366
Cluster13	8.0	0.6	0.2	0.0	Sinobacteraceae (Gammaproteobacteria); 219056
Cluster51	6.2	1.0	0.2	0.0	Bacillales (Firmicutes); 14393

Table 5. Changes in relative abundance of selected OTUs in the corn stover microbial community over time

Corn Stover: R12

<i>OTU</i>	<i>T1</i>	<i>T2</i>	<i>T3</i>	<i>T4</i>	<i>Taxonomy; greengenes ID (http://greengenes.lbl.gov)</i>
Cluster1	0.4	28.7	29.5	23.5	Microbispora (Actinobacteria); 12149
Cluster3	2.8	10.7	6.4	1.2	Paenibacillus (Firmicutes); 27158
Cluster8	0.1	5.8	6.2	0.5	Sphingomonadales (Proteobacteria); 180031
Cluster9	6.0	4.6	6.6	10.8	Acidobacteria; 113347

Corn Stover: R13

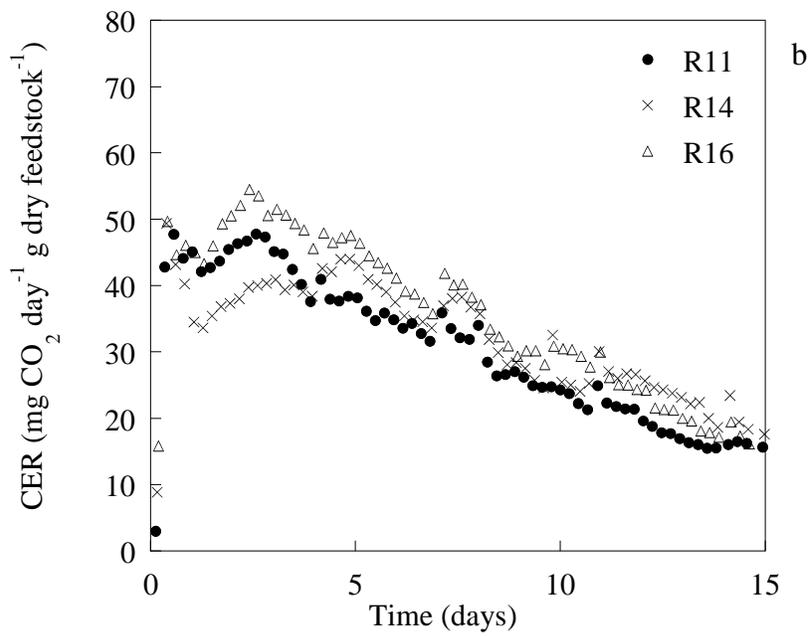
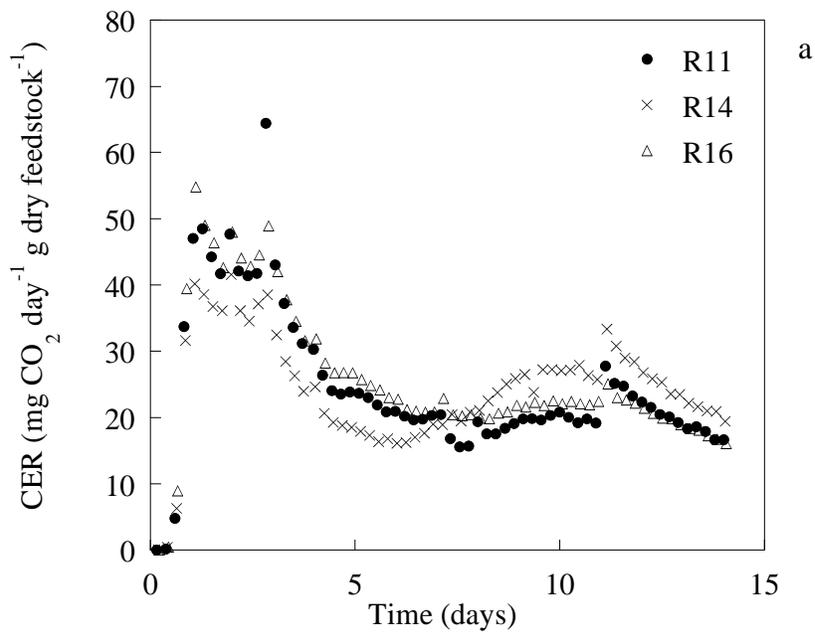
<i>OTU</i>	<i>T1</i>	<i>T2</i>	<i>T3</i>	<i>T4</i>	<i>Taxonomy; greengenes ID (http://greengenes.lbl.gov)</i>
Cluster1	0.5	8.0	36.0	30.2	Microbispora (Actinobacteria); 12149
Cluster5	7.2	6.9	2.8	20.6	Gammaproteobacteria; 210648
Cluster11	9.7	7.0	1.3	1.1	Dactylosporangium (Actinobacteria); 336366

Corn Stover: R15

<i>OTU</i>	<i>T1</i>	<i>T2</i>	<i>T3</i>	<i>T4</i>	<i>Taxonomy; greengenes ID (http://greengenes.lbl.gov)</i>
Cluster1	1.2	4.7	34.3	19.5	Microbispora (Actinobacteria); 12149
Cluster5	3.9	5.1	2.8	17.2	Gammaproteobacteria; 210648
Cluster11	12.2	8.8	0.9	0.4	Dactylosporangium (Actinobacteria); 336366

Table 6. SIMPER analysis between T4 microbial communities of higher-contributing (>75% cumulative contribution) OTUs.

<i>OTU</i>	<i>Average Abundance Switchgrass</i>	<i>Average Abundance Corn Stover</i>	<i>Contribution (%)</i>	<i>Taxonomy; greengenes ID (http://greengenes.lbl.gov)</i>
Cluster5	0.00	17.17	14.78	Gammaproteobacteria; 210648
Cluster3	15.79	2.00	11.87	Paenibacillus; 27158
Cluster4	14.88	3.61	9.70	Chelatococcus; 232942
Cluster6	10.12	0.40	8.37	Bacteroidetes; 270397
Cluster1	29.02	24.38	7.91	Microbispora (Actinobacterium); 12149
Cluster9	0.00	8.16	7.03	Acidobacteria; 113347
Cluster8	5.19	0.51	4.02	Sphingomonadales; 180031
Cluster30	0.28	3.76	2.99	Brevibacillus; 358085
Cluster14	5.64	3.46	2.96	Schlegelella; 99971
Cluster28	0.00	2.42	2.08	Gammaproteobacteria; 239420
Cluster46	0.00	1.78	1.53	Azospirillales; 150158
Cluster13	0.00	1.73	1.48	Gammaproteobacteria; 219056



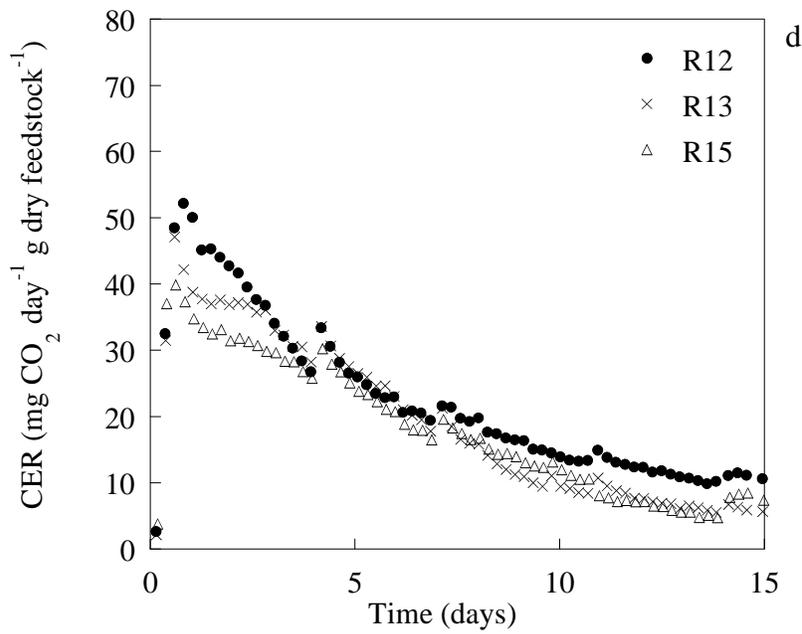
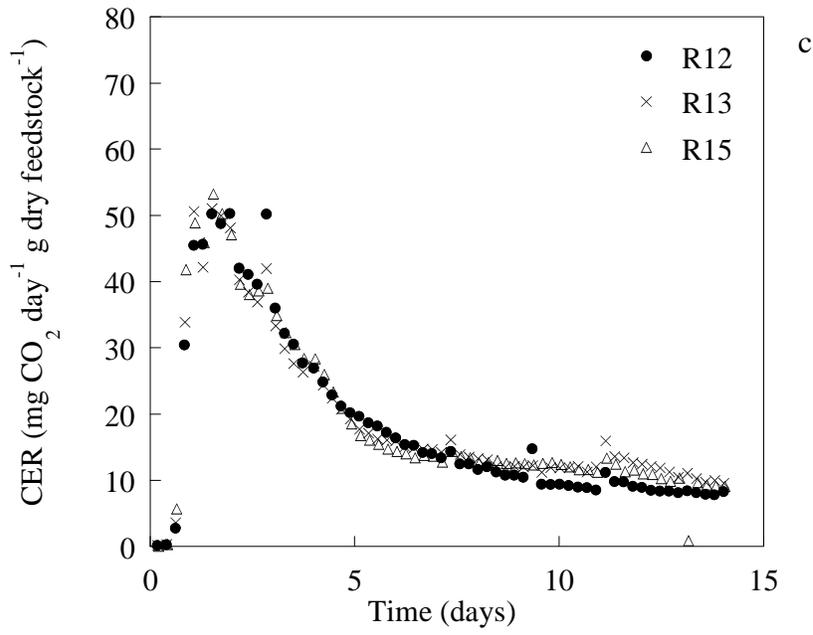
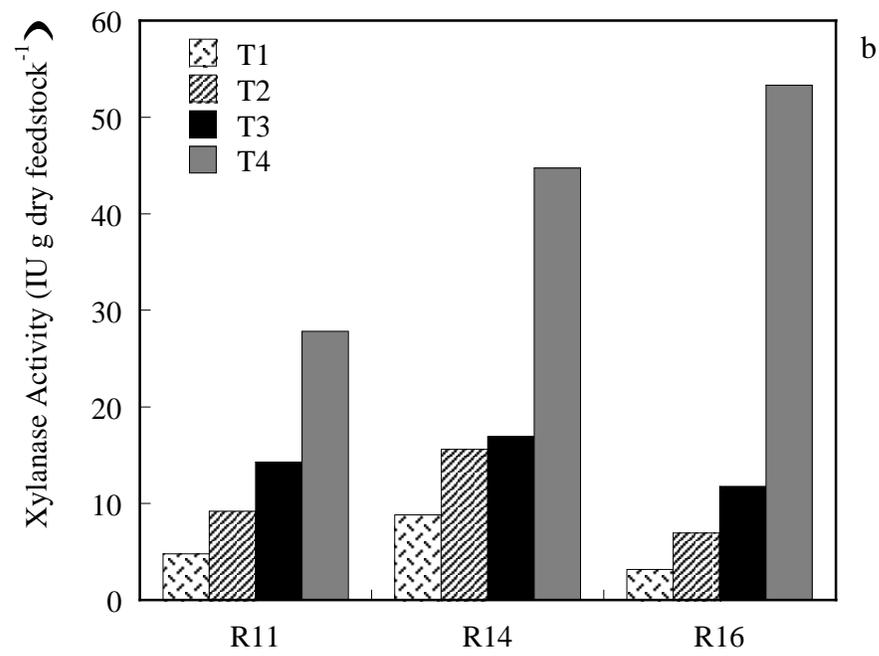
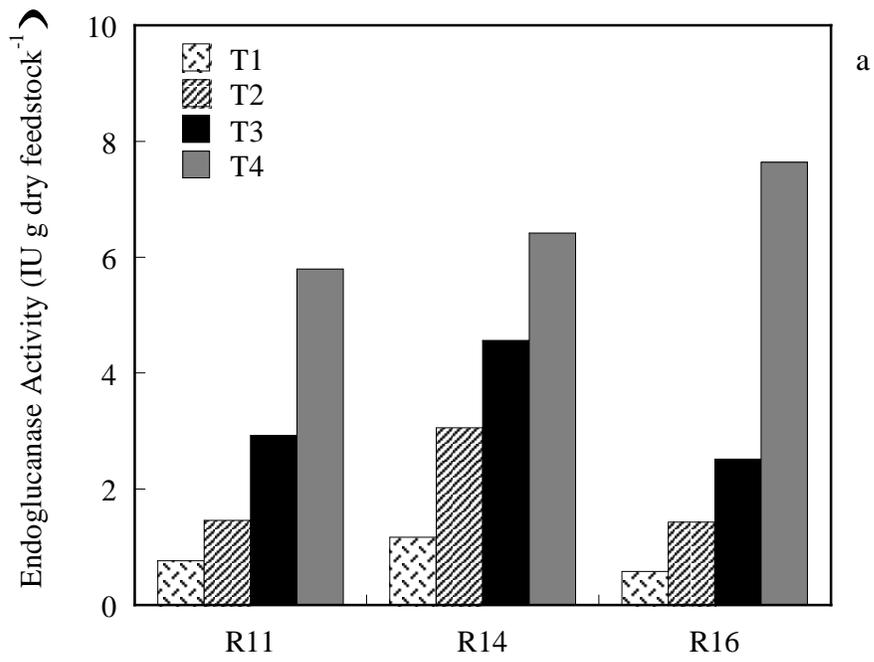


Figure 1. Carbon dioxide evolution rate profiles for the first (T1) and last (T4) two weeks of the enrichment study: a) switchgrass, T1, b) switchgrass, T4, c) corn stover, T1, d) corn stover, T4



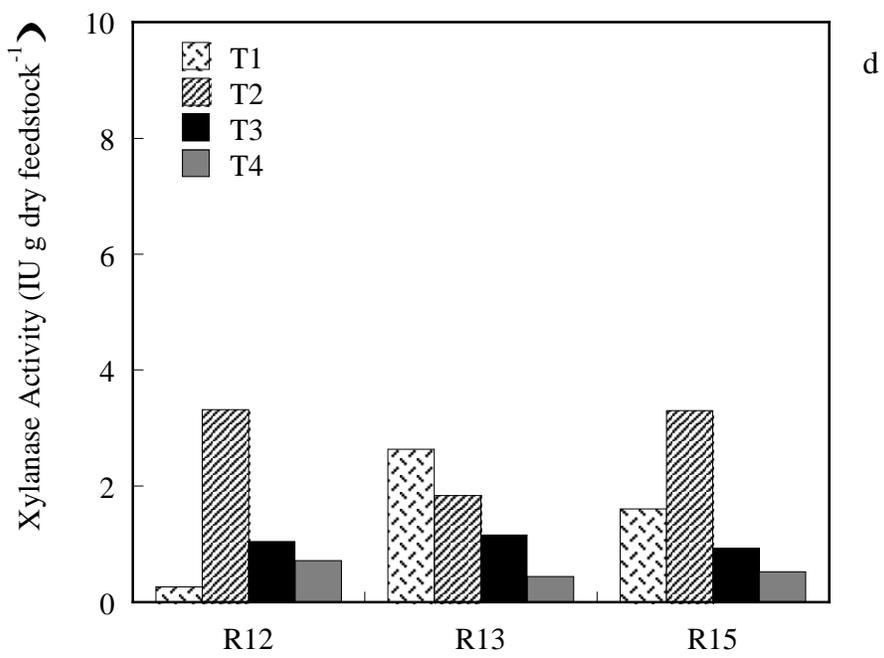
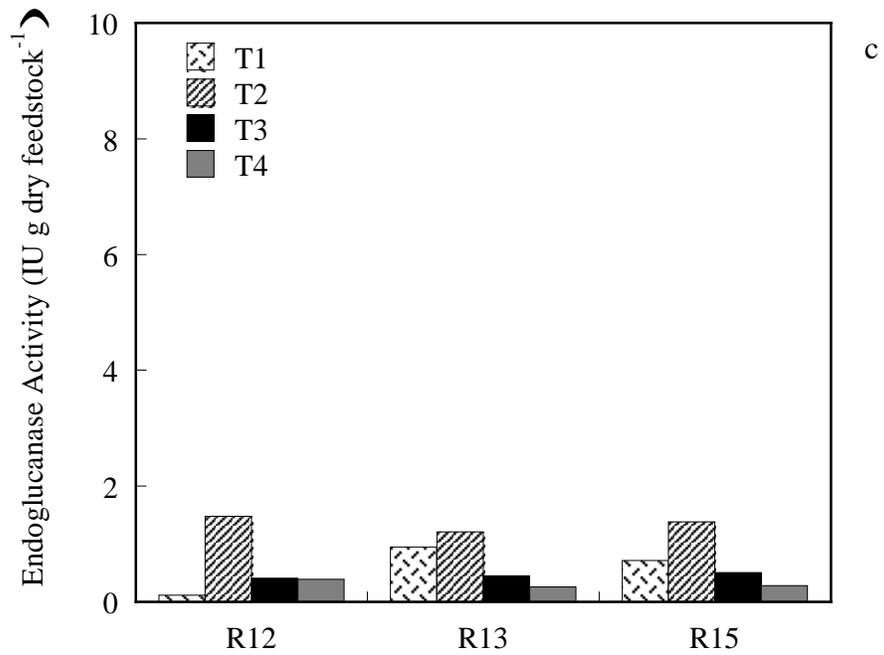
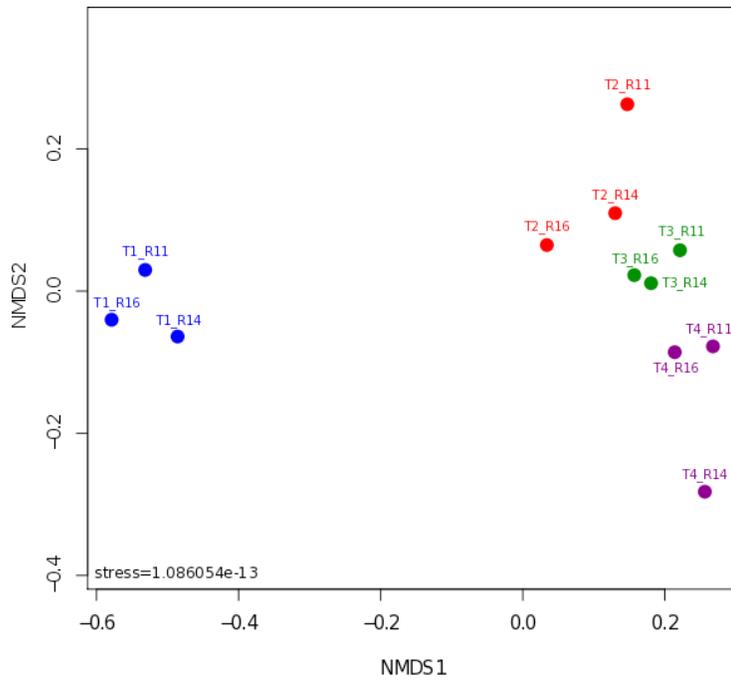


Figure 2. Enzyme activities at end of each two-week incubation period: (a) Endoglucanase activity on switchgrass, (b) Xylanase activity on switchgrass, (c) Endoglucanase activity on corn stover, and (d) Xylanase activity on corn stover

a



b

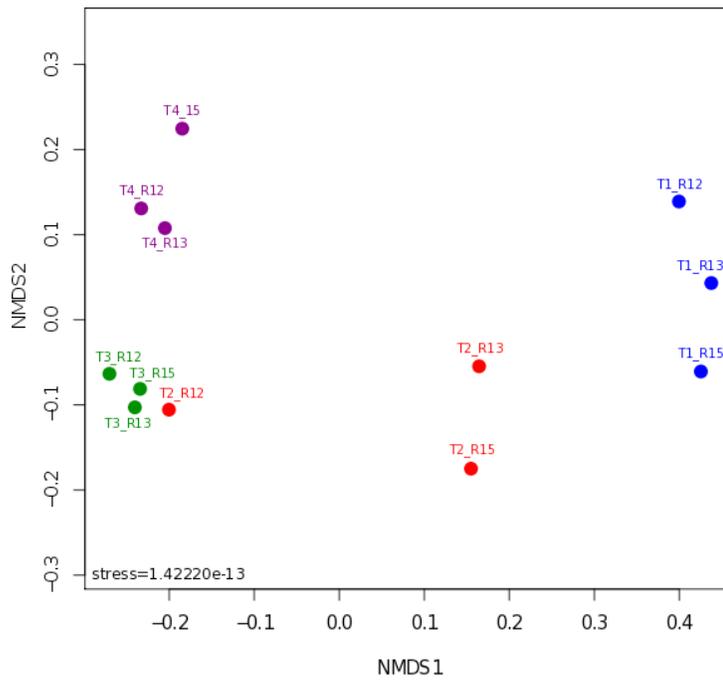


Figure 3. Shift of microbial community after each two-week incubation (T1 – T4). Non-metric multidimensional scaling (NMDS) of a) switchgrass and b) corn stover communities.

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