10-2016

Correlation Analysis of Enzyme activities and Deconstruction of Ammonia-pretreated Switchgrass by Bacterial-fungal Communities

Abhiney Jain

H. Sandra Pelle
Sacred Heart University

J. Michael Henson

Correlation analysis of enzyme activities and deconstruction of ammonia-pretreated switchgrass by bacterial-fungal communities

Abhiney Jain, Sandra H. Bediako, J. Michael Henson

PII: S0960-8524(16)31005-7
DOI: http://dx.doi.org/10.1016/j.biortech.2016.07.041
Reference: BITE 16803

To appear in: *Bioresource Technology*

Received Date: 20 April 2016
Revised Date: 7 July 2016
Accepted Date: 8 July 2016

Please cite this article as: Jain, A., Bediako, S.H., Michael Henson, J., Correlation analysis of enzyme activities and deconstruction of ammonia-pretreated switchgrass by bacterial-fungal communities, *Bioresource Technology* (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.07.041

Correlation analysis of enzyme activities and deconstruction of ammonia-pretreated switchgrass by bacterial-fungal communities

Authors: **Abhiney Jain[1], Sandra H. Bediako[2] and J. Michael Henson[3*]**

[1]Biotechnology Institute, University of Minnesota, Twin Cities, 1479 Gortner Avenue, Falcon Heights, MN 55108

[2] Sacred Heart University, Department of Biology, 5151 Park Avenue, Fairfield, CT 06825

[3]Department of Biological Sciences, 157A Life Science Facility, Clemson University, Clemson, SC 29634

***Corresponding Author: hhenson@clemson.edu, 864-656-3057, and 864-656-0435 (fax)**

Abstract

The mixed microbial communities that occur naturally on lignocellulosic feedstocks can provide feedstock-specific enzyme mixtures to saccharify lignocelluloses. Bacterial-fungal communities were enriched from switchgrass bales to deconstruct ammonia-pretreated switchgrass (DSG). Correlation analysis was carried out to elucidate the relationship between microbial decomposition of DSG by these communities, enzymatic activities produced and enzymatic saccharification of DSG using these enzyme mixtures. Results of the analysis showed that β-glucosidase activities and xylosidase activities limited the extent of microbial deconstruction and enzymatic saccharification of DSG. The results also underlined the importance of ligninase activity for the enzymatic saccharification of pretreated lignocellulosic feedstock. The bacterial-fungal communities developed in this research can be used to produce enzyme mixtures to deconstruct DSG, and the results from the correlation analysis can be used to optimize these enzyme mixtures for efficient saccharification of DSG to produce second-generation biofuels.

Keywords: Bacterial-fungal consortia; thermophilic; mesophilic; lignocellulose degradation; biofuels

1. Introduction

Switchgrass, *Panicum virgatum,* is generally stored as bales on an agricultural field after harvesting where it undergoes various degrees of decomposition by the naturally-occurring microorganisms present either from the field soils or on the switchgrass itself. The microorganisms involved with the decomposition of baled switchgrass biomass include members of both *Bacteria* and *Eukarya* domains. These bacteria and fungi can be utilized as a source for lignocellulolytic enzymes to convert the lignocellulosic switchgrass to soluble carbohydrates on the hypothesis that they have adapted to decompose switchgrass on the fields.

Switchgrass bales typically contain little free water when they are stored on the agricultural field. In essence, therefore, the microorganisms decompose the switchgrass in the switchgrass bales under solid-state culture conditions. An objective of this study is to enrich for switchgrass-degrading microbial consortia from switchgrass bales and assess their potential to produce lignocellulolytic enzymes. Therefore, it becomes important to develop and study the microbial enrichments under solid-state culture conditions to mimic their natural environment. This strategy should provide a more accurate insight into the functioning of the microbial consortia as they degrade switchgrass on the field.

Furthermore, the use of solid-state cultivation for production of enzymes has several advantages for scaling up of the industrial process. Solid-state cultivation studies have shown higher volumetric production of enzymes when compared to submerged-state cultivation (Hölker and Lenz, 2005; Krishna, 2005; Pandey *et al.*, 2000; Robinson *et al.*, 2001). The enzymes produced during solid-state cultivation were also shown to be more stable to changes in pH and temperature along with reduced risk of enzyme inhibition and protease degradation (Hölker and

Lenz, 2005; Krishna, 2005; Robinson *et al.*, 2001). Another advantage of solid-state cultivation is the reduction in water usage resulting in smaller volume bioreactors further reducing cost for construction materials and operations. Furthermore, downstream processing of products will be easier requiring less energy because of the reduced requirements to extract valuable products from reduced quantities of water (Krishna, 2005; Robinson *et al.*, 2001; Singhania *et al.*, 2009).

The conversion of plant biomass, like switchgrass, requires a variety of enzymes. The major enzymes required to decompose plant biomass include cellulases, hemicellulases and ligninases (Lynd *et al.*, 2002) that act upon the three major components of any plant biomass, cellulose, hemicellulose and lignin, respectively. In addition, a wide range of auxillary enzymes, such as esterases and pectinases, are also required to efficiently decompose the plant biomass. The complex structure of lignocellulosic biomass, however, may not allow all the enzymes present in a mixture access to their respective substrates. Lignin can serve especially as a barrier for the cellulolytic enzymes to bind to cellulose and hemicellulose (Kumar and Wyman, 2009) thus decreasing the saccharification efficiency of plant biomass to soluble carbohydrates. Plant biomass, therefore, is initially pretreated to remove lignin. By reducing the amount of lignin present, the cellulases and hemicellulases are provided more access to their respective substrates (Kumar and Wyman, 2009). One such pretreatment technology used to remove lignin is ammonia soaking pretreatment (Sherman *et al.*, 2012). While pretreatment technologies are able to remove lignin from plant biomass, a significant amount may remain after pretreatment.

An optimized enzyme mixture for saccharification of plant biomass requires a better understanding of the relationships between the various enzyme activities and the hydrolysis of the pretreated biomass. This study reports the results from the enrichment of switchgrass-degrading bacterial-fungal consortia collected from switchgrass bales stored under field

conditions. These consortia were the source of enzyme mixtures that were employed to convert delignified switchgrass to soluble sugars. Correlation analyses were performed between the enzyme activities and microbial decomposition of switchgrass, as well as between enzyme activities and enzymatic saccharification. Results of these correlation analyses contribute to better understand the process of converting pretreated switchgrass by microbial consortia and their enzyme mixtures.

2. Materials and methods

2.1. Collection of switchgrass samples

Switchgrass samples were collected in February 2011 from several switchgrass bales that had been harvested and baled in 2009, 2010 and 2011 at Clemson University's Pee Dee Research and Education Center (Florence, SC, USA). Samples were returned to the Clemson University laboratory and stored at room temperature. A total of 12 switchgrass samples were collected from switchgrass bales.

2.2. Enrichment of bacterial-fungal consortia

Switchgrass samples were used as inocula for enrichment of naturally occurring microbial consortia. Duplicate enrichments were initiated at both mesophilic and thermophilic temperature of 25 °C and 50 °C, respectively, resulting in 24 enrichments at each temperature. Enrichments were established under solid-state conditions to mimic natural conditions by inoculating 0.1 g (wet weight) of a switchgrass sample onto 1 g of autoclaved delignified switchgrass (DSG) moistened with 2 mL of sterile medium at an initial pH of 6.5 in sterile Petri plates. Modified Czapek Dox medium was used and is composed of the following (g/L deionized water*):* NH_4NO_3, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCL, 0.5; and $FeSO_4$, 0.01. DSG was

prepared as a growth substrate by pretreating switchgrass with ammonia to reduce the lignin content as previously described (Jain et al. 2013). The pretreated switchgrass was then stored at 4 °C until use. The delignified switchgrass (DSG) obtained using this method contained 36.5% glucan and 20.5% xylan, respectively (Jain et al., 2013). Studies were performed in absence of light.

After incubation for 10 days, the Petri plates showing visual microbial growth were selected for further enrichment by aseptically transferring the DSG covered with microorganisms. Before every transfer, the DSG on each Petri plate was thoroughly mixed using a sterile spatula to reduce microbial heterogeneity. The procedure was repeated after each 10-day incubation period for 10 transfers at which time the enrichment cultures were deemed to be stable. The enriched consortia were then screened for their capability to use DSG as the sole carbon source by measuring the dry weight loss of DSG after a period of incubation. Aliquots of 0.1 g of the enrichment cultures were used to inoculate the 10 mL screw-cap tubes containing 1 g of autoclaved DSG moistened with 2 mL of sterile medium at an initial pH of 6.5. Additional screw-cap tubes were inoculated with autoclaved 0.1 g of DSG, instead of enrichment cultures, as control tubes to account for abiotic dry weight loss of DSG during the incubation period. The tubes were incubated at either 25 °C or 50 °C for 10 days. DSG dry weight loss was measured by washing the DSG remaining after growth with distilled water and centrifuging at 1500 rpm for 15 min. This procedure was repeated three times after which the pellet was dried at 80 °C for 48 hours before being weighed. The dry weight loss of DSG caused by different enrichments was calculated as relative dry weight loss with respect to the abiotic dry weight loss.

2.3. Extraction of enzymes

The enzymes were extracted from the tubes inoculated with different enrichments using the procedure of Reddy *et al.* (2011). Each tube was added with 10 mL of extraction mixture containing 49% ethylene glycol (weight/weight), 1% sodium chloride (weight/weight), 0.01% Tween-80 (weight/weight) and 50% of distilled water. The tubes were thoroughly mixed and incubated at 25 °C for an hour. After incubation, the enzyme solution was centrifuged at 10,620 × *g* for 5 min. Supernatant was aseptically filtered through a sterile 0.45 µm nylon filter and the filtrate was collected. The buffer components in the filtrate were exchanged with sodium citrate buffer (0.05M, pH 5) using VivaSpin columns with 5 KDa molecular weight cut off (VivaSpin 20 MWCO 5000, GE Healthcare Life Sciences) and the enzyme mixture thus obtained was used for evaluation of the different enzyme activities.

2.4. Enzyme Activity Assays

Exoglucanase activity was evaluated as Avicelase activity whereas endoglucanase activity was evaluated as carboxymethyl cellulase (CMCase) activity. Hemicellulase activity was evaluated as xylanase activity. Pectinase activity was measured as polygalacturonidase activity. CMCase and xylanase activity were measured using the method described by Reddy *et al.* (2011). Briefly, to evaluate CMCase activity 40 µL of the supernatant was added to 40 µL of 2% carboxymethyl cellulose in citrate buffer (0.05 M, pH 5) on PCR plates, sealed and incubated at 50 °C for 30 min in a thermocycler (Eppendorf, Mastercycler ep gradient). After incubation, 80 µL of dinitrosalicylic acid (DNS) reagent (Ghose *et al.*, 1987) was added to the wells and sealed before being at incubated at 95 °C for 5 min. The PCR plate was allowed to cool to room temperature and 100 µL of solution from each well was transferred to new 96-well plate. The

absorbance was measured at 540 nm using a plate reader (EON™, BioTek Instruments, Inc., VT, USA,). Xylanase activity was evaluated similarly except that supernatant was added to 1% birchwood xylan (Sigma-Aldrich Co., MO, USA) in 0.05 M citrate buffer (pH 5) and incubated at 50 °C for 10 min prior to addition of DNS and measuring absorbance as above. Avicelase activity was evaluated using the same protocol except that 1% Avicel in citrate buffer (0.05 M, pH5) was used as the substrate and the mixture was incubated for 60 min. For the above enzyme assays, one unit of enzyme activity is defined as 1 µmol of either glucose or xylose released per mL of enzyme per min.

Pectinase activity was measured using 1% polygalacturonic acid solution as the substrate and the mixture was incubated for 30 min using the same protocol as used for CMCase assay. The galacturonic acid released was measured using the DNS assay as described above for CMCase activity. One unit of pectinase activity is defined as 1 µmol of galacturonic acid released per mL of enzyme per min.

β-glucosidase activity was measured using a protocol adapted from German *et al.* (2012) using a 200 µL reaction containing 128 µL of 0.05 M citrate buffer (pH 5), 32 µL of enzyme mixture and 40 µL of 4-methylumbiliferone-β-D-glucopyranoside solution (6.77 mg/100 mL distilled water; Sigma-Aldrich) as substrate. The mixture was incubated for 30 min at 50 °C and then the fluorescence resulting from the release of 4-methylumbiliferone (MUB) as a result of β-glucosidase activity was measured using a plate reader (Synergy H1) at excitation wavelength of 365 nm and emission wavelength of 450 nm. Chitinase and xylosidase activities were evaluated using the same protocol except for different substrates. The substrate used for chitinase assay was 4-MUB-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich), whereas the substrate used for

xylosidase assay was 4-MUB-β-D-xylopyranoide (Sigma-Aldrich). One unit of enzyme activity is defined as 1 nmol of MUB released per milliliter of enzyme per min.

Ligninase activities were measured as phenol oxidase and peroxidase activities. Phenol oxidase activity and peroxidase activity were measured in a 200 µL reaction in 96 well plates. Phenol oxidase activity was measured by mixing 100 µL of 25 mM of L-dihydroxy phenylalanine (L-DOPA) with 100 µL of the enzyme mixture that was allowed to incubate for four hours after which the absorbance was taken at 460 nm. Peroxidase activity was evaluated using the same protocol except that the substrate used consisted of 25 mM L-DOPA with 0.3% hydrogen peroxide. Phenol oxidase and peroxidase activity are expressed as the change in absorbance at 460 nm per hour.

Total protein concentration in the supernatant was measured using the Bradford assay (Bradford, 1976). The specific activities for different enzymes were calculated by dividing the enzyme activity for each enzyme by the total protein measured in the enzyme solution as prepared in Section 2.3.

2.5. Microbial diversity assessment

DNA was extracted from the cultures using MoBio soil DNA extraction kit (MoBio). PCR-denaturing gradient gel electrophoresis (DGGE) was used to assess the diversity in the microbial community present in the different enrichments. PCR-DGGE analysis was performed for bacterial communities by electrophoresing the V3 region of 16S rRNA gene amplified using a nested PCR protocol. The first step of the nested PCR was performed using 1492R and 8f primers in 50 µL reactions with 1 µL (10 µM) forward and reverse primers, 5 µL of template DNA, and 25 µL of GoTaq® Green Master Mix (Promega) using a Mastercycler ep gradient

(Eppendorf) with the following protocol: initial denaturation at 95 ºC for 10 min followed by 30 cycles of denaturation at 95 ºC for 45 sec, annealing at 55 ºC for 45 sec, and extension at 72 ºC for 1min followed by a final extension at 72 ºC for 5 min. The PCR product obtained was purified using QIAquick PCR purification kit (Qiagen) and used as the template for the second step. The second step of the nested PCR was performed to amplify the V3 region of 16S rRNA gene using PRBA 338F and PRUN 518R (Ovreås *et al.*, 1997) primers using the following protocol: 9 min of initial denaturation at 95 ºC, followed by 30 cycles containing denaturation step at 94 ºC for 30 sec, annealing stage at 55 ºC for 30 sec, an extension at 72 ºC for 30 sec and a final extension at 72 ºC for 7 min. PCR amplifications were carried out in 50 µL reactions with 1 µL (10 µM) forward and reverse primers, 5 µL of template DNA, and 25 µL of GoTaq® Green Master Mix (Promega).

PCR-DGGE analysis was performed for fungal communities by electrophoresing the ITS region gene that was amplified using a nested PCR protocol. The first step of the PCR amplification was performed using ITS1F (Gardes and Bruns, 1993) as the forward primer and ITS4 (White *et al.,* 1990) as the reverse primer in a 50 µL reaction using the following protocol: initial denaturation at 98 ^{o}C for 30 sec followed by 30 cycles of denaturation at 98 ^{o}C for 10 sec, annealing at 55 ^{o}C for 20 sec, and extension at 72 ^{o}C for 1 min, with a final extension of 7 min at 72 ^{o}C. PCR amplifications were carried out in 50 µL reactions with 1 µL (10 µM) forward and reverse primers, 5 µL of template DNA, and 25 µL of GoTaq® Green Master Mix (Promega). The PCR product obtained was purified using QIAquick PCR purification kit (Qiagen). The second step of the amplification was carried out using the purified DNA from the first step. The second step of the amplification was performed using ITS5 (White *et al.,* 1990) as the forward primer and ITS2 (White *et al.,* 1990) as the reverse primer using the following protocol: initial

denaturation at 98 ^{o}C for 30 sec followed by 30 cycles of denaturation at 98 ^{o}C for 10 sec, annealing at 55 ^{o}C for 20 sec, and extension at 72 ^{o}C for 1 min, with a final extension of 7 min at 72 ^{o}C. PCR amplifications were carried out in 50 µL reactions with 1 µL (10 µM) forward and reverse primers, 5 µL of template DNA, and 25 µL of GoTaq® Green Master Mix (Promega). The PCR product was purified using QIAquick PCR purification kit (Qiagen). DNA concentrations were measured using Quant-iT™ PicoGreen® dsDNA reagent kit according to the manufacturer's instructions (Life Technologies, USA) and 250 ng of the PCR product was electrophoresed for each community.

PCR products were resolved (Bio-Rad DCode) using an 8% (w/v) polyacrylamide gel in 1 X TAE buffer using a 35% to 60% denaturing gradient for bacterial community analysis and 20% to 60% denaturing gradient for fungal community analysis, where 100% denaturant contains 7 M urea and 40% formamide. Electrophoresis was carried out at 20 V for 10 min, then 50 V for 17 hours. DGGE gels were stained for 60 min with SYBR® Safe DNA gel stain in 1 X TAE and viewed under UV transillumination (Life Technologies, USA).

For the analysis of DGGE gel, the band profile for different enrichments was converted into a binary matrix based on presence or absence of the bands. The binary matrix was analyzed using BiodiversityR package in R (Kindt and Coe, 2008).

2.6. Enzymatic saccharification of ammonia pretreated switchgrass

Enzymatic saccharification of DSG was carried out in 10 mL reactions in 50 mL plastic BD Falcon tubes™. The reaction mixture contained 5 mL of the enzyme mixture, 5 mL of 0.05 M citrate buffer (pH 5) and 0.1 g (dry weight) of DSG. The reaction mixture was incubated at 50 ºC for 24 hours while shaking at 200 rpm for 24 hours. After incubation, samples were collected

and analyzed for the release of respective sugars using high performance liquid chromatography (HPLC) (Jain *et al.* 2014).

The data including dry weight loss, enzyme activities and amount of sugars produced during enzymatic saccharification for the mesophilic and thermophilic microbial enrichments were compared in multiple comparisons using two-tailed unpaired Student's t-test. Pearson's correlation coefficient, r, was calculated to assess the correlations between different parameters reported in this study.

3. Results and discussion

Fifteen of the initial 48 switchgrass microbial enrichments were able to sustain growth after 10 successive transfers. Nine grew at 25 ºC whereas six grew at 50 ºC. Percent dry weight loss of DSG was measured after 10 days incubation under solid-state culture conditions with the results shown in Fig. 1a for the stable mesophilic enrichments and Fig. 1b for the stable thermophilic enrichments. These data are presented as relative dry weight loss as they were calculated with respect to results of abiotic controls. Overall, the mesophilic enrichments produced a lower relative dry weight loss when compared to the thermophilic enrichments. Mesophilic enrichments MC6 and MC3 showed greater relative dry weight loss amongst mesophilic enrichments while thermophilic enrichments TD2 and TB2 showed greater relative dry weight loss amongst thermophilic enrichments.

3.1. PCR-DGGE analysis

PCR-DGGE analysis performed on the sustained enrichments showed considerable differences in the bacterial and fungal communities within mesophilic and thermophilic enrichments. Greater diversity was observed among the bacterial communities in the enrichments

when compared to the fungal communities, which were observed to be much less diverse. Also, there was higher diversity found among bacterial communities in mesophilic enrichments when compared to the thermophilic enrichments. The fungal and bacterial communities were clustered into separate clades based on temperature, as shown in Fig. 2a and Fig. 2b, with exceptions of fungal community present in MC2 that was present in the same clade as the fungal communities for thermophilic enrichments.

3.2. Enzyme activities produced by the mesophilic and thermophilic enrichments during microbial deconstruction of DSG

The stable enrichments were assessed for the production of different enzymes required to decompose switchgrass. The enzyme activities assessed include Avicelase (exoglucanase), CMCase (endoglucanase), xylanase (hemicellulase), phenol oxidase and peroxidase (ligninase), pectinase, β-glucosidase, and xylosidase. Chitinase activities were also measured to evaluate the possible effects that bacteria might have on the fungal population. Results of the various enzyme activities produced by the mesophilic and the thermophilic enrichments are shown in Fig. 3a through 3f. The data from enzyme activities produced by different microbial enrichments were grouped into either mesophilic or thermophilic for analysis, based on whether the respective microbial enrichment was mesophilic or thermophilic. Thermophilic enrichments produced higher β-glucosidase activities (alpha=0.05, p=0.0005) and higher xylosidase activities (alpha=0.05, p=0.0006) compared to mesophilic enrichments. The thermophilic enrichments also produced almost five times the phenol oxidase activity compared to the mesophilic enrichments. There were no statistically significant differences observed between mesophilic and thermophilic enrichments for the other enzyme activities measured in this study. Table 1 shows the specific enzyme activities (enzyme activity/mg total extracellular protein) for different enrichments. The

thermophilic enrichments, TC4 and TA3 produced significantly higher specific activities, except the phenol oxidase activity for TC4, as compared to the other enrichments.

3.3. Correlations between enzyme activities and dry weight loss during microbial degradation of DSG

Correlation coefficients were calculated between the dry weight loss for different bacterial-fungal enrichments and their respective enzyme activities (Table 2). The correlation coefficients were calculated for mesophilic and thermophilic enrichments separately and for the enrichments pooled together. Mesophilic enrichments showed positive correlation, except for avicelase and phenol oxidase activities, for different enzyme activities as compared to the thermophilic enrichments (Table 2). The mesophilic and thermophilic enrichments grouped separately, as well as the enrichments pooled together showed positive correlation with the CMCase activity.

3.4. Enzymatic saccharification of DSG

DSG was saccharified using the enzyme mixture collected from the solid-state culture of bacterial-fungal enrichments. As shown in Fig. 4, enzyme mixtures obtained from thermophilic enrichments released a greater amount of sugars from DSG compared to the amount of sugar released using mesophilic enrichments enzyme mixtures (alpha=0.05, p=0.00007). The mesophilic microbial enrichment MC6 was an exception to this result. Enzyme mixtures from thermophilic enrichments released a greater amount of xylose from DSG compared to glucose (alpha=0.05, p=0.000002). Enzymes collected from thermophilic enrichments released greater amount of xylose from DSG compared to the release of xylose using mesophilic enrichments enzyme mixtures (alpha=0.05, p=0.000007). The mesophilic microbial enrichment MC6 was an

exception to this result. No significant difference was observed for the amount of glucose released from DSG using enzyme mixtures from mesophilic and thermophilic enrichments.

3.5. Correlation between dry weight loss during microbial degradation of DSG and sugars released during enzymatic saccharification of DSG

There was a positive correlation (r = 0.55) between the dry weight loss observed for bacterial-fungal enrichments and the total amount of sugar released when DSG was saccharified using enzymes extracted from these enrichments (Fig. 5). Also, there was a positive correlation (r = 0.57) between the dry weight loss observed for bacterial-fungal enrichments and the concentration of xylose released from DSG using the respective enzyme mixtures. However, there was no correlation (r = 0.05) between the dry weight loss observed for different bacterial-fungal enrichments and the concentration of glucose released from DSG using the respective enzyme mixtures.

Similar results were obtained when data from mesophilic enrichments and thermophilic enrichments were pooled separately. The correlation between dry weight loss and total sugars released during enzymatic saccharification, however, was much higher for mesophilic enrichments (r = 0.54) compared to that for thermophilic enrichments (r = 0.32). Again, there was positive correlation between the dry weight loss and the amount of xylose released for both mesophilic (r = 0.63) and thermophilic enrichments (r = 0.45), whereas there was no correlation between the dry weight loss and the amount of glucose released for either thermophilic and mesophilic enrichments.

3.6. Correlation between enzyme activities and sugars released during enzymatic saccharification of DSG

As shown in Table 3, a strong correlation was observed between the total concentrations of sugars released from DSG using enzymes mixtures collected from different bacterial-fungal enrichments and β-glucosidase activity. Though there was no correlation between β-glucosidase activity and the concentration of glucose released from DSG. Interestingly, there was very strong correlation observed between β-glucosidase activity and the concentration of xylose released. A strong correlation was observed between the total sugars released from DSG and xylosidase activity. Xylosidase activity also showed strong correlation with the concentration of xylose released and no correlation with the concentration of glucose released during enzymatic saccharification. The two ligninase activities, phenol oxidase and peroxidase activities also showed positive correlations with the total concentration of sugar released and the concentration of xylose released from DSG during its enzymatic saccharification.

The bacterial-fungal microbial communities were developed under solid-state culture conditions to mimic the natural environmental conditions under which switchgrass bales are decomposed on the field. The study was aimed to understand the enzymatic factors affecting the decomposition of switchgrass bales on the field and then to use that knowledge to design enzyme mixtures for efficient enzymatic saccharification of switchgrass for production of soluble carbohydrates. Selection of the microbial community for the production of enzyme mixtures to saccharify the switchgrass, therefore, is an important step in this overall process. No single enzyme activity can be used to screen for the microbial community because the lignocelluloses are decomposed by a concerted action of a number of different enzymes (Santhanam *et al*., 2012).

Total dry weight loss of DSG observed by the microbial enrichments was used as the parameter to select for the microbial enrichment to produce the mixture of lignocellulolytic enzymes. A positive correlation was observed between total dry weight loss and total sugars released from DSG during enzymatic saccharification, which supports that dry weight loss can be used as a credible parameter to screen for a microbial enrichment to produce enzyme mixture for DSG saccharification. Though the r and r^2 values (Fig. 5) obtained show moderate to weak relationship between the dry weight loss, during DSG decomposition by microbial enrichments, and the amount of sugars released during enzymatic saccharification of DSG. This emphasizes that dry weight loss can only be used as a qualitative parameter for initial screening of the microbial enrichments, and it cannot be used for the purpose of predictive modeling of enzymatic saccharification of DSG by the microbial enrichments. The positive correlation between the dry weight loss and the amount of sugars released during enzymatic saccharification of DSG was dependent on the amount of xylose released during enzymatic saccharification. For mesophilic enrichments, the two enrichments that showed highest dry weight loss, MC6 and MC3, also showed significantly higher release of xylose than glucose when enzyme mixtures were used for saccharification. The incubation time of ten days used in this study may have selected for hemicellulose-degrading microbial communities. A longer incubation time during the enrichment process may have selected for the microbial community that can saccharify the cellulosic fraction efficiently. Also, a longer incubation time may be required for the same microbial community to produce more cellulolytic enzymes.

The dry weight loss of DSG during microbial degradation and the sugars released during enzymatic saccharification of DSG showed higher correlation with the β-glucosidase and xylosidase activities when compared to other enzyme activities measured in this study. This

result suggests that the product inhibition of cellulases and xylanases may play a major role during the decomposition of lignocelluloses in the environment and during enzymatic saccharification of lignocelluloses. Cellobiose has been shown to inhibit cellulases (Gruno *et al.*, 2004), whereas xylose has been shown to inhibit the endoxylanases (Khanna and Gauri, 1993). Xylo-oligomers have been shown to be the stronger inhibitors of cellulases than cellobiose (Kumar and Wyman, 2009; Quing and Wyman, 2011). β-glucosidase and xylosidase remove these feedback inhibitions by converting cellobiose and xylo-oligomers to glucose and xylose, respectively. In the environment, microorganisms consume the cellobiose and xylo-oligomers as lignocelluloses are decomposed whereas during the enzymatic saccharification of lignocelluloses cellobiose and xylo-oligomers accumulate when β-glucosidase and xylosidase activities are either low or absent. These observations may explain our findings that the correlation between β-glucosidase and xylosidase activities and the amount of sugars released during enzymatic saccharification was much higher when compared to their correlation with the dry weight loss during incubation of DSG with the microbial enrichments.

The lignin present in the lignocelluloses is believed to reduce the accessibility of enzymes to cellulose and xylans, thus reducing the effectiveness of the enzymes because of unproductive binding to lignin (Kumar and Wyman, 2009; Chang and Holtzapple, 2000; Mansfield *et al*., 1999). The thermophilic enrichments showing significantly higher ligninase activity than the mesophilic enrichments might explain the higher dry weight loss of DSG by the thermophilic enrichments. The importance of ligninase activities during the microbial decomposition or enzymatic saccharification of the lignocelluloses is further emphasized by the results showing positive correlation between the ligninase activities and the concentration of sugars released during the enzymatic saccharification of DSG.

4. Conclusions

The bacterial-fungal microbial communities enriched from switchgrass bales were able to produce enzymes mixtures required for microbial deconstruction and enzymatic saccharification of DSG to produce second-generation biofuels. The results showed that both the microbial decomposition and enzymatic saccharification were limited by β-glucosidase and xylosidase activities. The analysis further emphasizes the importance of ligninases during the decomposition of pretreated biomass, like DSG, used in this study. This result emphasizes the need to study the effect of addition of ligninases in the enzyme mixtures used for saccharification of pretreated biomass for production of soluble carbohydrates.

Acknowledgements

US Department of Energy award number DE-FG36-08GO88071 funded this research. The authors acknowledge Dr. Jim Frederick at the Clemson University Pee Dee Research and Education Center, near Florence, SC, where the switchgrass was grown and harvested. This material is based upon work supported by NIFA/USDA under project number SC-1700474 and is Technical Contribution No. 6403 of the Clemson University Experiment Station.

References

1. Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.
2. Chang, V. S., Holtzapple, M. T., 2000. Fundamental factors affecting biomass enzymatic reactivity. In Twenty-First Symposium on Biotechnology for Fuels and Chemicals, Humana Press 5-37.
3. Gardes, M., Bruns, T. D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2(2), 113-118.
4. German, D. P., Marcelo, K. R., Stone, M. M., Allison, S. D., 2012. The Michaelis–Menten kinetics of soil extracellular enzymes in response to temperature: a cross-latitudinal study. Glob. Change Biol. 18, 1468-1479.
5. Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257-268.
6. Gruno, M., Väljamäe, P., Pettersson, G., Johansson, G., 2004. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol. Bioeng. 86, 503-511.
7. Hölker, U., Lenz, J., 2005. Solid-state fermentation—are there any biotechnological advantages? Curr. Opin. Microbiol. 8, 301-306.
8. Jain, A., Morlok, C. K., Henson, J. M., 2013. Comparison of solid-state and submerged-state fermentation for the bioprocessing of switchgrass to ethanol and acetate by *Clostridium phytofermentans*. Appl. Microbiol. Biotechnol. 97, 905-917.
9. Jain, A., Hammonds, R. E., Kerrigan, J. L., Henson, J.M., 2014. Characterization of a *Trichoderma atroviride* strain isolated from switchgrass bales and its use to saccharify

ammonia-pretreated switchgrass for biobutanol production. Biomass Bioenergy 64, 299-308.

10. Khanna, S., Gauri, 1993. Regulation, purification, and properties of xylanase from *Cellulomonas fimi.* Enzyme Microb. Technol. 15, 990-995.
11. Krishna, C., 2005. Solid-state fermentation systems-an overview. Crit. Rev. Biotechnol. 25, 1-30.
12. Kumar, R., Wyman, C. E., 2009. Cellulase adsorption and relationship to features of corn stover solids produced by leading pretreatments. Biotechnol. Bioeng. 103, 252-267.
13. Kindt, R., Coe, R., 2008. BiodiversityR: GUI for biodiversity and community ecology analysis.
14. Lynd, L. R., Weimer, P. J., Van Zyl, W. H., Pretorius, I. S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506-577.
15. Mansfield, S. D., Mooney, C., Saddler, J. N., 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol. Prog. 15, 804-816.
16. Ovreås, L., Forney, L., Daae, F. L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63(9), 3367-3373.
17. Pandey, A., Soccol, C. R., Mitchell, D., 2000. New developments in solid state fermentation: I-bioprocesses and products. Process Biochem. 35, 1153-1169.
18. Qing, Q., Wyman, C. E., 2011. Hydrolysis of different chain length xylooliogmers by cellulase and hemicellulase. Bioresource Technol., 102, 1359-1366.

19. Reddy, A. P., Allgaier, M., Singer, S. W., Hazen, T. C., Simmons, B. A., Hugenholtz, P., VanderGheynst, J. S., 2011. Bioenergy feedstock-specific enrichment of microbial populations during high-solids thermophilic deconstruction. Biotechnol. Bioeng. 108, 2088-2098.

20. Robinson, T., Singh, D., Nigam, P., 2001. Solid-state fermentation: a promising microbial technology for secondary metabolite production. Appl. Microbiol. Biotechnol. 55, 284-289.

21. Santhanam, N., Badri, D. V., Decker, S. R., Manter, D. K., Reardon, K. F., Vivanco, J. M., 2012. Lignocellulose decomposition by microbial secretions. In Secretions and Exudates in Biological Systems, Springer Berlin Heidelberg, 125-153.

22. Sherman, S. R., Goodell, J. J., Milliken, C. E., Morris, J. A., Gorensek, M. B., 2012. A new process developed for separation of lignin from ammonium hydroxide pretreatment solutions. Environ. Prog. Sustain. Energy 31, 130-138.

23. Singhania, R. R., Patel, A. K., Soccol, C. R., Pandey, A., 2009. Recent advances in solid-state fermentation. Biochem. Eng. Journal 44, 13-18.

24. White, T. J., Bruns, T., Lee, S. J. W. T., Taylor, J. W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18(1), 315-322.

Figure Legends

Fig. 1. Mean dry weight loss of DSG for bacterial-fungal enrichments after incubation for 10 days (a) mesophilic enrichments (b) thermophilic enrichments. The error bars are represented by standard errors. The microbial enrichments labelled with same letter resulted in statistically similar dry weight loss.

Fig. 2. (a) Principal Coordinate Analysis (PCoA) plot for bacterial communities present in the bacterial-fungal enrichments (b) cluster analysis for fungal communities present in the bacterial-fungal enrichments.

Fig. 3. Enzyme activities for different bacterial-fungal enrichments (a) Avicelase and CMCase (b) xylanase (c) β-glucosidase and xylosidase (d) phenyl oxidase and peroxidase (e) pectinase (f) chitinase. Error bars are represented by standard errors. The microbial enrichments labelled with same letter produced statistically similar enzyme activity.

Fig. 4. Concentration of sugars released when DSG was saccharified for 24 hours using enzymes collected from the different bacterial-fungal enrichments. The microbial enrichments labelled with same letter resulted in statistically similar sugar concentration.

Fig. 5. Relation between the dry weight loss observed for different bacterial-fungal enrichments and the sugars released from DSG using the enzyme mixtures collected from bacterial-fungal enrichments.

Fig. 1a

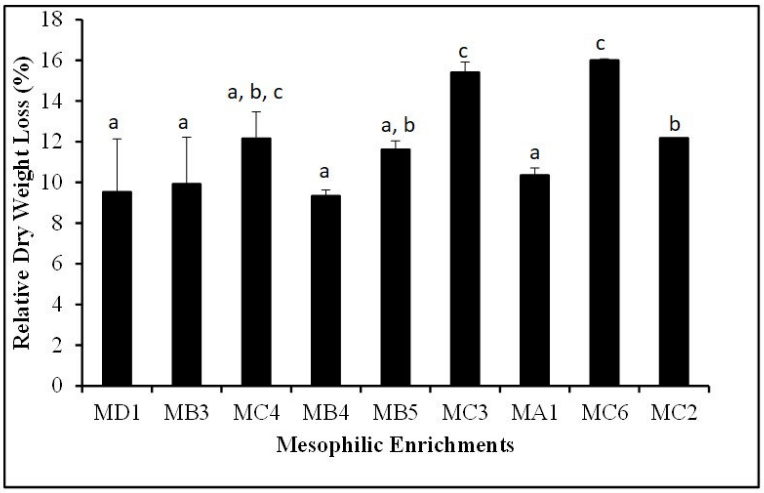

Fig.1b

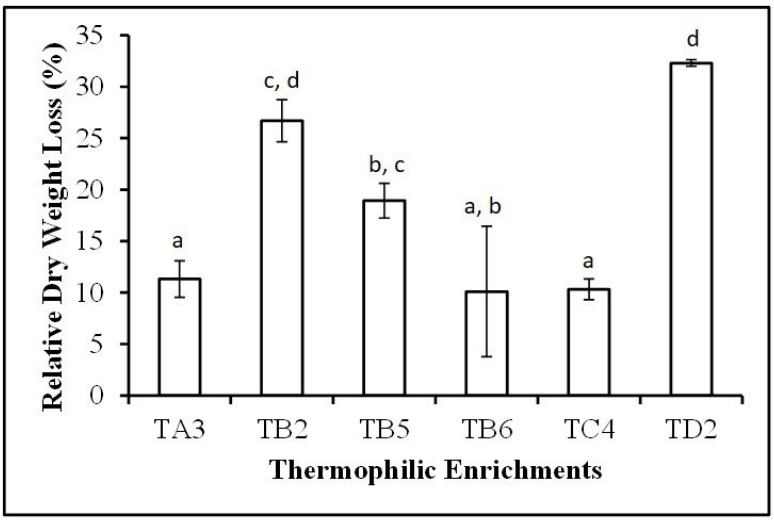

Fig. 2a

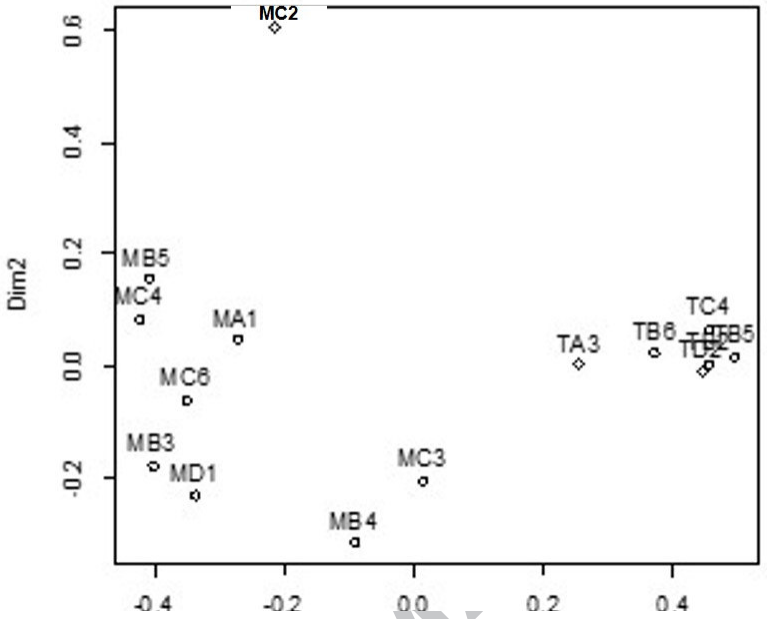

Fig.2b

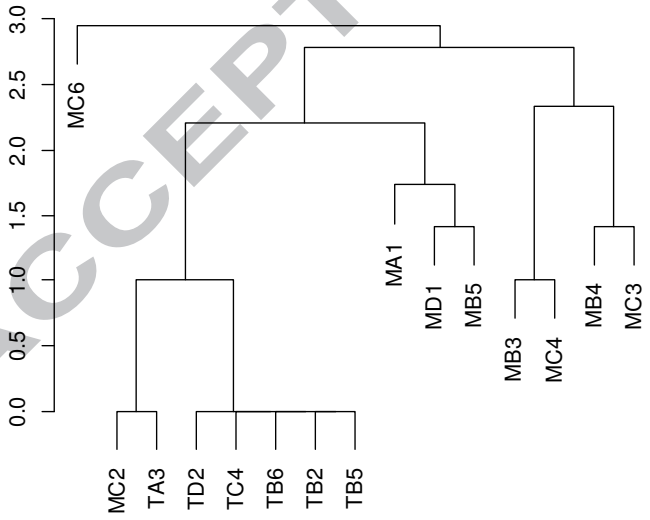

Fig. 3a

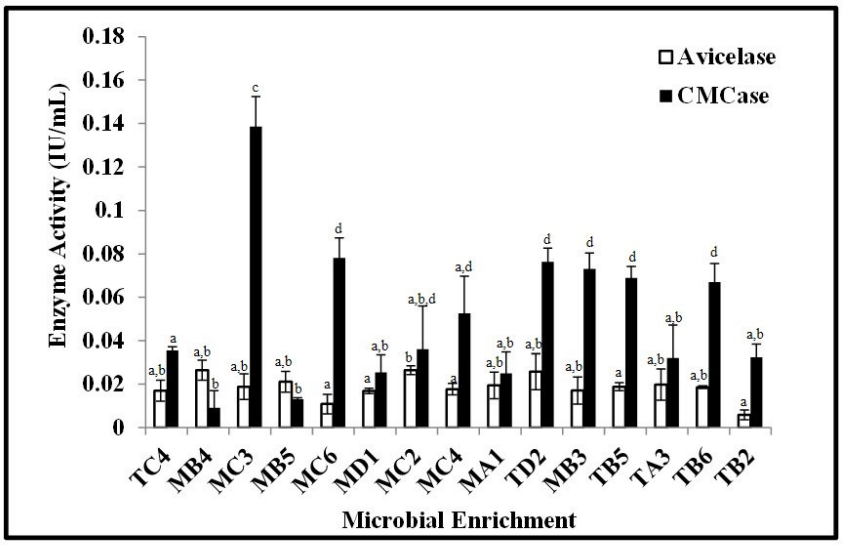
Avicelase
CMCase
Enzyme Activity (IU/mL)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
TC4
MB4
MC3
MB5
MC6
MD1
MC2
MC4
MA1
TD2
MB3
TB5
TA3
TB6
TB2
Microbial Enrichment

Fig. 3b

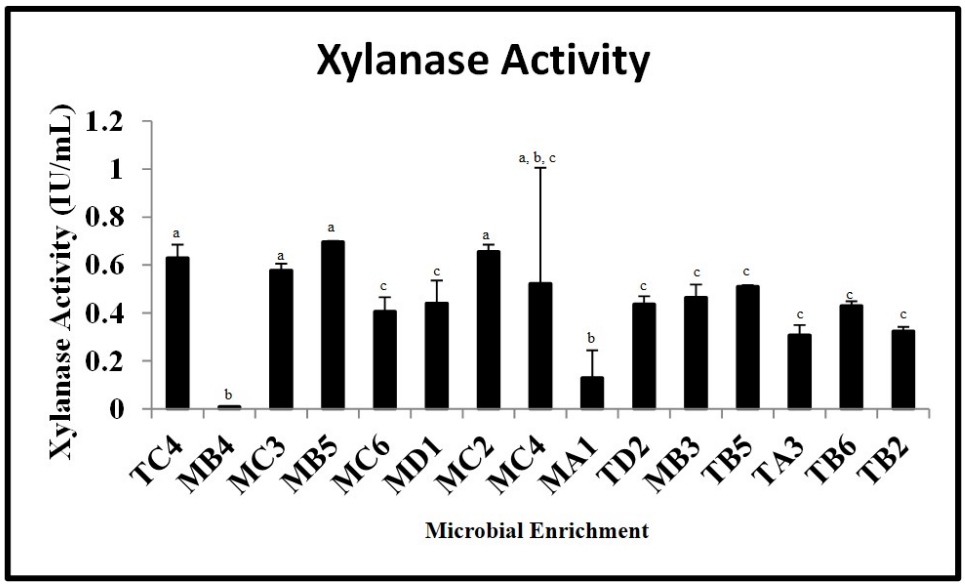
Xylanase Activity
Xylanase Activity (IU/mL)
1.2
1
0.8
0.6
0.4
0.2
0
TC4
MB4
MC3
MB5
MC6
MD1
MC2
MC4
MA1
TD2
MB3
TB5
TA3
TB6
TB2
Microbial Enrichment

Fig. 3c

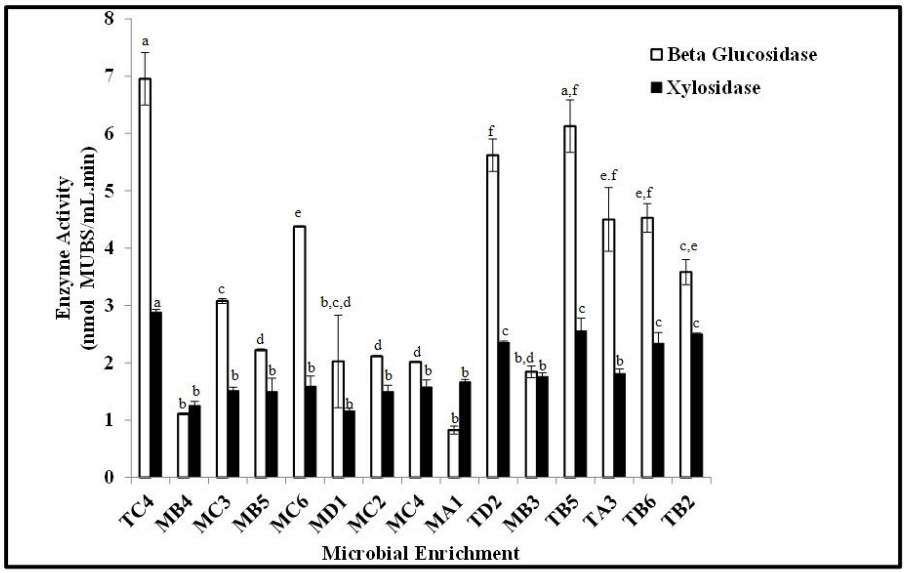
Beta Glucosidase
Xylosidase
Enzyme Activity (nmol MUBS/mL.min)
Microbial Enrichment
TC4
MB4
MC3
MB5
MC6
MD1
MC2
MC4
MA1
TD2
MB3
TB5
TA3
TB6
TB2

Fig. 3d

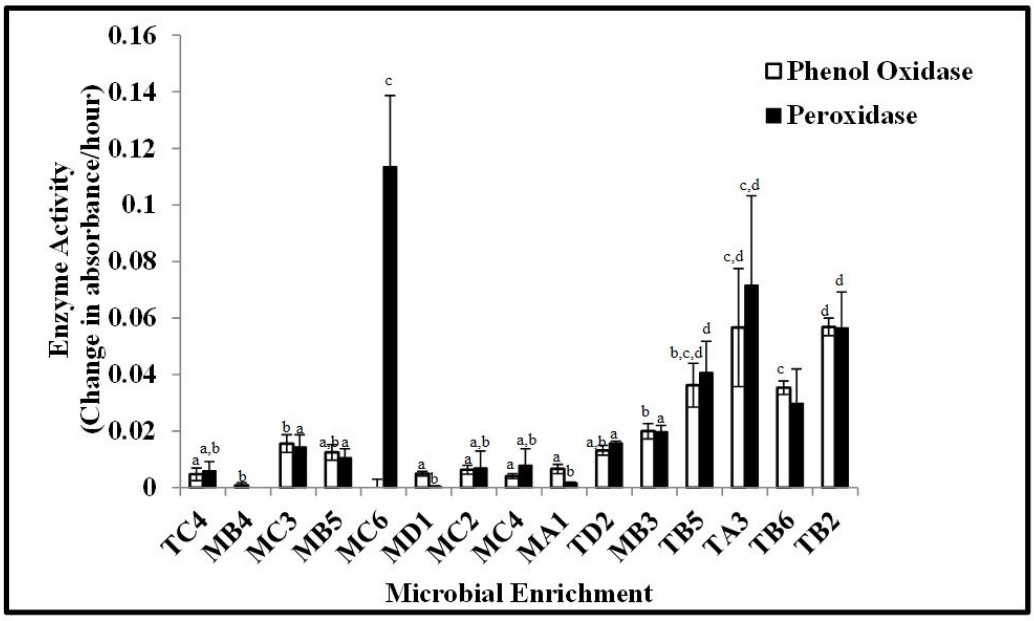
Phenol Oxidase
Peroxidase
Enzyme Activity (Change in absorbance/hour)
Microbial Enrichment
TC4
MB4
MC3
MB5
MC6
MD1
MC2
MC4
MA1
TD2
MB3
TB5
TA3
TB6
TB2

Fig. 3e

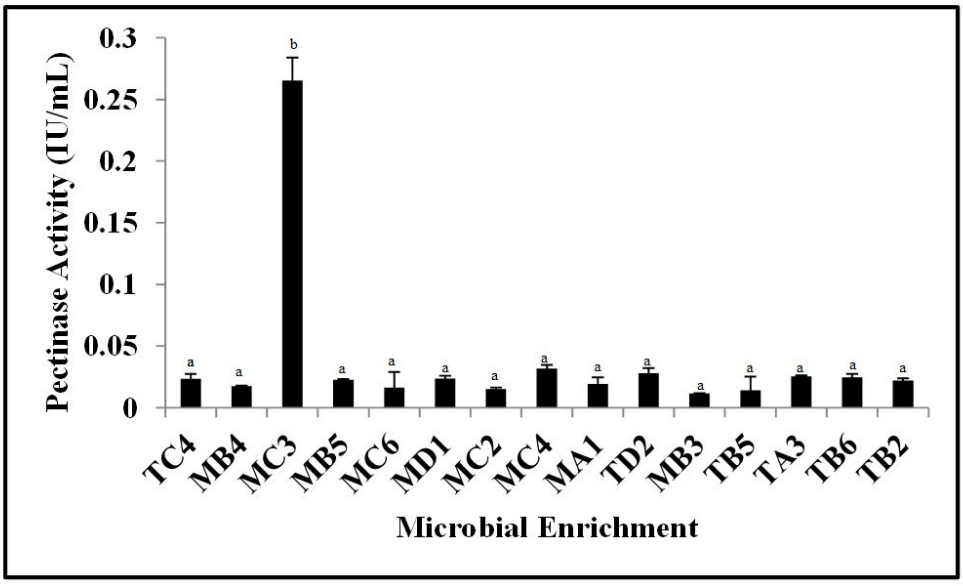
Pectinase Activity (IU/mL)
0.3
0.25
0.2
0.15
0.1
0.05
0
TC4 MB4 MC3 MB5 MC6 MD1 MC2 MC4 MA1 TD2 MB3 TB5 TA3 TB6 TB2
Microbial Enrichment

Fig. 3f

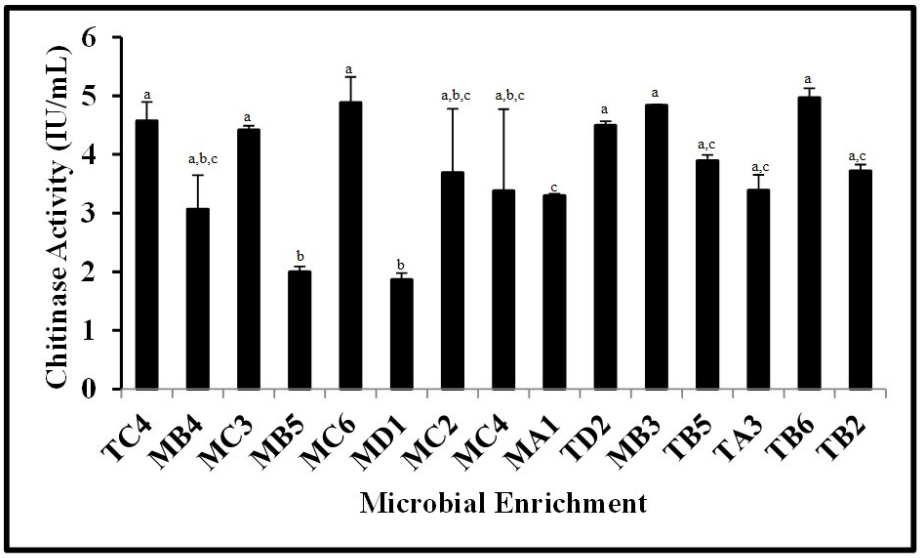
Chitinase Activity (IU/mL)
6
5
4
3
2
1
0
TC4 MB4 MC3 MB5 MC6 MD1 MC2 MC4 MA1 TD2 MB3 TB5 TA3 TB6 TB2
Microbial Enrichment

Fig. 4

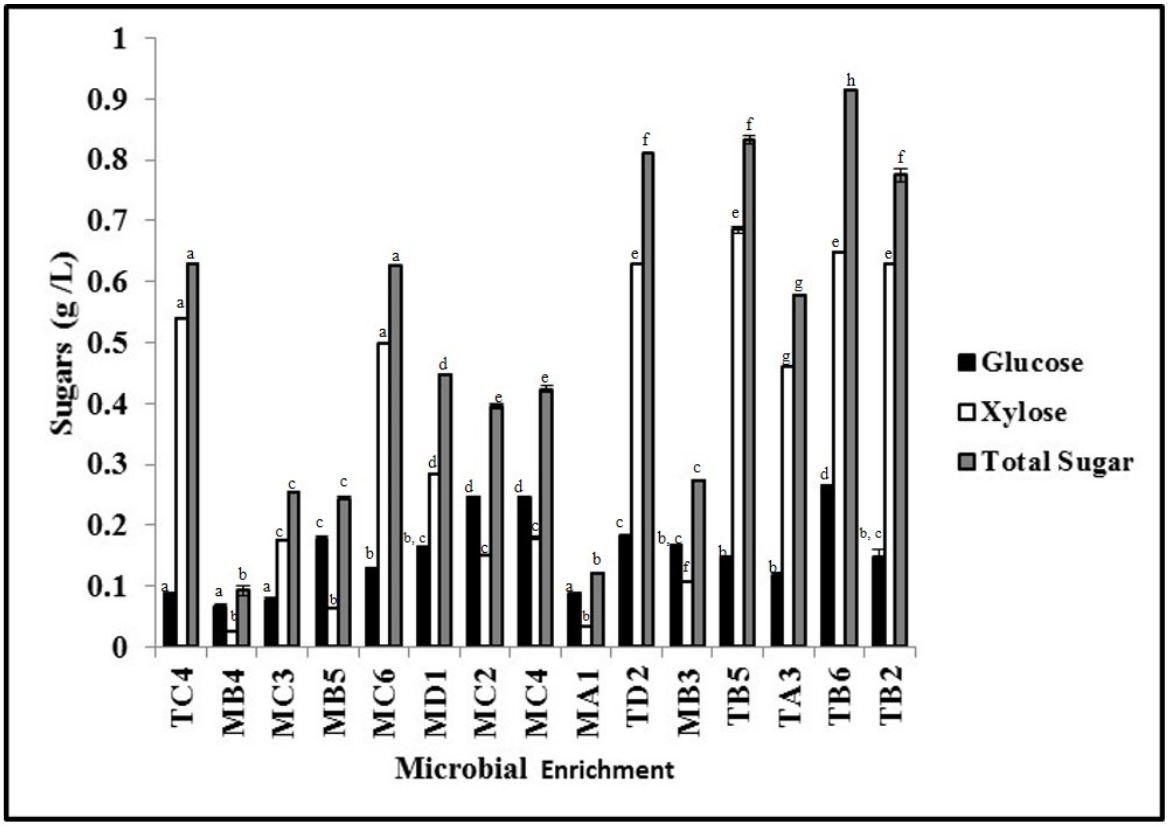

Fig.5

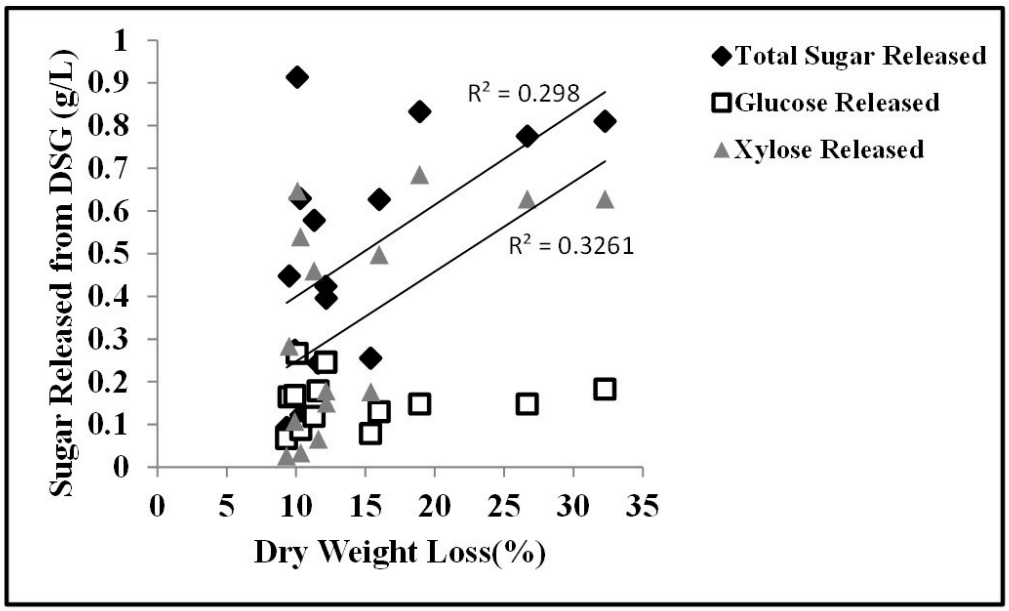

Table Legends

Table 1. Specific enzyme activities produced by different bacterial-fungal enrichments. Avicelase, CMCase, xylanase and pectinase activities are presented as IU mg^{-1} protein; β-glucosidase, xylosidase and chitinase activities are presented as nmole MUBS min^{-1} mg^{-1} protein; phenyl oxidase and peroxidase activities are presented as change in absorbance $hour^{-1}$ mg^{-1} protein.

Table 2. Correlation coefficients between different enzyme activities and the dry weight loss for the bacterial-fungal enrichments.

Table 3. The correlation coefficients between different enzyme activities and the sugars released during enzymatic saccharification of DSG using enzyme mixtures collected from different bacterial-fungal enrichments.

Tables

Table 1.

Enzyme	TC 4	MB 4	MC 3	MB 5	MC 6	MD 1	MC 2	MC 4	MA 1	TD 2	MB 3	TB 5	TA 3	TB 6	TB 2
Avicelase	0.17	0.06	0.03	0.05	0.02	0.05	0.15	0.05	0.05	0.07	0.03	0.04	0.2	0.07	0.02
CMCase	0.35	0.02	0.22	0.03	0.15	0.07	0.20	0.14	0.06	0.20	0.15	0.16	0.34	0.27	0.09
β-Glucosidase	69.37	2.66	5.07	4.77	8.60	5.83	11.94	5.54	2.02	14.51	3.68	14.61	48	18.14	9.92
Xylanase	6.28	0.02	0.95	1.50	0.80	1.23	3.70	1.26	0.31	1.13	0.93	1.22	3.27	1.72	0.90
Xylosidase	28.76	3.01	2.50	3.22	3.12	3.35	8.45	4.34	4.07	6.07	3.51	6.11	19.33	9.36	6.93
Phenol Oxidase	0.047	0.002	0.026	0.026	0	0.014	0.036	0.011	0.016	0.034	0.040	0.086	0.604	0.142	0.158
Peroxidase	0.060	0	0.024	0.023	0.222	0.001	0.039	0.022	0.004	0.041	0.040	0.097	0.764	0.120	0.156
Pectinase	0.78	0.14	1.46	0.16	0.11	0.23	0.29	0.29	0.16	0.11	0.07	0.11	0.43	0.33	0.08
Chitinase	45.59	7.36	7.27	4.29	9.59	5.37	20.81	9.30	8.05	11.60	9.66	9.28	36.13	19.91	10.30

Table 2.

Enzyme Activity Measured	Mesophilic Enrichments	Thermophilic Enrichments	Pooled Mesophilic and Thermophilic Enrichments
Avicelase	-0.45	-0.01	-0.17
CMCase	0.73	0.34	0.30
β-Glucosidase	0.88	-0.18	0.43
Xylanase	0.42	-0.26	0.04
Xylosidase	0.30	0.06	0.46
Phenol Oxidase	-0.04	-0.03	0.31
Peroxidase	0.69	-0.06	0.27
Pectinase	0.55	0.11	0.06
Chitinase	0.56	-0.13	0.26

Table 3.

Enzyme Activity	Glucose Released	Xylose Released	Total Sugars
Avicelase	**0.09**	**-0.38**	**-0.34**
CMCase	**0.01**	**0.24**	**0.22**
β-Glucosidase	**-0.02**	**0.88**	**0.81**
Xylanase	**0.42**	**0.14**	**0.23**
Xylosidase	**0.03**	**0.79**	**0.75**
Phenol Oxidase	**0.07**	**0.54**	**0.52**
Peroxidase	**-0.07**	**0.52**	**0.47**
Pectinase	**-0.31**	**-0.16**	**-0.23**
Chitinase	**0.05**	**0.46**	**0.45**

Highlights

- Bacterial-fungal consortia deconstructing ammonia pretreated switchgrass (DSG).
- Consortia produced cellulases, xylanases, pectinases, chitinases and ligninases.
- Correlation analysis conducted on enzyme activities and deconstruction of DSG.
- β-glucosidase and xylosidase activities limited deconstruction of DSG.
- Ligniniase activity plays an important role during deconstruction of DSG.