Farm Deployable Microbial Bioreactor for Fuel Ethanol Production

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Contributors: Benedict Okeke, Ananda Nanjundaswammy and Rosine Hall

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SUMMARY

Research was conducted to develop a farm and field deployable microbial bioreactor for bioethanol production from biomass. Experiments were conducted to select the most efficient microorganisms for conversion of plant fiber to sugars for fermentation to ethanol. Mixtures of biomass and surface soil samples were collected from selected sites in Alabama black belt counties (Macon, Sumter, Choctaw, Dallas, Montgomery, Lowndes) and other areas within the state of Alabama. Experiments were conducted to determine the effects of culture parameters on key biomass saccharifying enzymes (cellulase, beta-glucosidase, xylanase and beta-xylosidase). A wide-scale sampling of locally-grown fruits in Central Alabama was embarked to isolate potential xylose fermenting microorganisms. Yeast isolates were evaluated for xylose fermentation. Selected microorganisms were characterized by DNA based methods. Factors affecting enzyme production and biomass saccharification were examined and optimized in the laboratory. Methods of biomass pretreatment were compared. Co-production of amylolytic enzymes with celluloytic-xylanolytic enzymes was evaluated; and co-saccharification of a combination of biomass, and starch-rich materials was examined. Simultaneous saccharification and fermentation with and without pre-saccharifcation was studied. Whole culture broth and filtered culture broth simultaneous saccahrifcation and fermentation were compared. A bioreactor system was designed and constructed to employ laboratory results for scale up of biomass saccharification.

Substantial levels of cellulolytic and xylanolytic enzymes were observed in cultures of the fungal isolates. Four fungal strains, SG2, SG4, FS22A and FS5A produced promising levels of cellulolytic-xylanolytic enzymes. The hypercellulolytic mutant, RUT-C30 produced the highest cellulase activity. Cellulolytic activity of strains SG2, SG4, FS22A and FS5A are considerably high since they are wild-type strains that have not been subjected to strain improvement. SG2 and SG4 produced about half of the cellulolytic activity of the hypercellulolytic mutant strain RUT-30. Interestingly SG2 and SG4 produced more beta-glucosidase and beta-xylosidase than the commercial hypercellulolytic mutant strain RUT-C30. Synergistic interaction of different cellulolytic-xyanolytic enzymes is required for efficient conversion of lignocelluloses to sugars and subsequent fermentation to ethanol. Thus SG2 and SG4 are promising strains for biomass conversion to sugars and bioethanol.

Over 100 yeast isolates from mixtures of biomass and surface soil samples as well as fruit samples were examined for xylose conversion to ethanol. Xylose fermenting yeasts isolated from fruits displayed higher ethanol content compared to those from SDB. Based on ethanol content of cultures, twenty yeast isolates were shortlisted for further evaluation of xylose conversion to ethanol. Ethanol levels in xylose fermentation cultures were however low and not promising. Pigmented yeast isolates were designated potential lipid producing strains.

DNA based characterization of rvealed the identity of four promising fungal isolates to be *Trichoderma* (*Hypocerea*) sp. SG2, *Trichoderma* (*Hypocerea*) SG4, *Penicillium species* FS22A, *Trichoderma amazonicum (Hypocrea lutea)* FS5A. Two xylose fermenting yeast, *Debaryomyces pseudopolymorphus* YS28 and *Meyerozyma guilliermondii* (*Pichia* sp.) were also identified as potential catalysts for fermentation of lignocellulose hydrolysate. However, studies on xylose fermentation to ethanol by the selected isolates compared to *Pichia stipitis* showed that they were less effective than *Pichia stipitis*.

Biomass saccharification studies in flask experiments revealed the following: About 0.75g of glucose was saccharified from 20g of virgin switchgrass at 24h of incubation with crude enzyme and 25% of a commercial enzyme recommended amount. This would result in 7.5g of sugar from 200g of switchgrass. About 0.5 g of xylose was saccharified from 20g of virgin switchgrass at 24h of incubation with crude enzyme and 25% of a commercial enzyme dosage. This would result in 5.0g of sugar from 200g of switchgrass. From 200g of virgin switchgrass there is a potential to obtain a total of 12.5g of total sugar consisting of both glucose and xylose. Assuming that virgin switchgrass contains 40% cellulose (NREL, 2004), 200g of the material would contain 80g of cellulose and this would result in 7.5g of glucose. A combination of crude and 50% of a commercial enzyme resulted in 5.61g of glucose from 20g of acid pretreated switchgrass at 48h. This indicates a potential yield of 56.10g of glucose from 200g of acid pretreated biomass. A combination of crude enzyme extract and 50% commercial enzyme will result in 1.64g of xylose from 20g of acid pretreated biomass. This indicated a potential yield of 16.4g of xylose from 200g of acid pretreated switchgrass. Acid pretreated switchgrass has the potential to result in a total of 72.5g of total sugars consisting of glucose and xylose from 200g. Autoclaved switchgrass when treated with combination of crude and 50% commercial enzyme resulted in 2.18g of glucose from 20g of switchgrass. This indicates a potential yield of 21.8g of glucose from 200g of switchgrass. Autoclaved switchgrass when treated with combination of crude and 25 % or 50% of a commercial enzyme resulted in 1.00g of xylose from 20g of switchgrass. This indicates a potential yield of 10.0g of xylose from 200g of switchgrass. There is a potential to obtain about 31.8g of total sugar (glucose and xylose) from 200g of autoclaved switchgrass.

Among the three acid-pretreated biomass materials namely switchgrass, gammagrass and sawdust, switchgrass yielded the highest sugar when crude enzymes from SG2 or FS22A and their combinations with commercial enzyme were used and will be the choice of substrate for further testing. Supplementing SG2 or FS22A crude aqueous enzyme extract (N) with 50% of recommended commercial enzyme (C) dosing (C50N) or at 25% (C25N) resulted to higher sugar yield from switchgrass, suggesting 50-75% potential saving in biomass enzyme cost. In 10ml reaction volume in 100ml flasks, the glucose yield after 72h of saccharification was highest in C50N and yielded 5.61g/L followed by 5.11g/L in C25N and decreased in the following order(C50N > C25N > N > C). The ethanol yield was the highest in C50N at 2.12g/L, 1.7g/L in C25N, 1.2g/L in N and 1g/L in C with ethanol conversion efficiencies of 74%, 65%, 55% and 52% respectively, suggesting that residual nutrients in enzymes prepared in the laboratory (N) may have boosting effect in biomass saccharification/fermentation compared to the commercial enzyme used independently. Studies on ethanol fermentation of pretreated switchgrass saccharified with C50N of SG2 showed that after 24h fermentation of 1L sample in 5L bottle, 2.0g/L ethanol was produced with a conversion efficiency of about 80%. At the beginning of fermentation, there was 4.97g/L of glucose and 1.37g/L xylose respectively. Based on the saccharification of acid-pretreated switchgrass samples with different combinations of SG2 crude and commercial enzymes followed by 24h ethanol

fermentation in flasks and large media bottles, glucose release of 4.97 to 5.6g/L and xylose release of 1.37 to 1.64g/L is possible from C50N leading to ethanol production of 2.0 to 2.12g/L with an ethanol conversion efficiency ranging from 74% to 80%. Our flask experiment achieved 2.0g of ethanol from 4.97g of glucose using *S. cerevisiae* which theoretically corresponds 40g of ethanol from 100g of glucose. In another flask experiment 2.12g ethanol was obtained from 5.61g of glucose which theoretically corresponds to about 38g of ethanol from 100g of glucose. Our new yeast isolates underperformed in xylose conversion to ethanol.

Of the five different feedstock concentrations (2%, 5%, 10%, 15% and 20%) tested, 5% yielded the highest sugars upon saccharification with SG2 crude enzyme. Although 10% showed the overall highest sugar in 72h; the corresponding yields at 24h and 48h were lesser than that for 2% and 5%. Higher concentrations of 15% and 20% make the medium too thick which does not allow mixing and the final product has little or no liquid for sampling and caused data variation. Presence of liquid is vital for proper enzyme action on the feedstock. So using high substrate concentrations is ruled out. Feedstock concentrations of 2% or 5% seem to be suited well for saccharification. Although 5% gave the highest sugar yield, the yield is not proportionately increasing when compared with 2% and higher concentration.

Acid-pretreated paper powder at 5% concentration yielded 10g/L glucose after 48h. Accordingly, white-paper waste generated can be utilized for saccharification and potential biofuel production. It could be used to supplement switchgrass feedstock. White paper with and without print was used in this experiment. Untreated paper yielded negligible sugar whereas acid pretreatment was able to yield substantial sugars with SG2 crude enzyme. Supplementation of paper powder to switchgrass feedstock at 50:50 to get a total of 6% resulted in high glucose and xylose levels which was slightly greater than that obtained at 5% switchgrass alone.

The sugars obtained by repeated saccharification of saccharified-switchgrass did not result in high sugar levels. The data obtained when compared with regular saccharification for 72h showed the following: Glucose from 2% switchgrass after 72h regular saccharification is 9.4g/L whereas cumulative yield after repeated extraction is 7.5g/L. Glucose from 5% switchgrass after 72h regular saccharification is 11.7g/L whereas the cumulative yield after repeated saccharification is 13.3g/L. Xylose yield from 2% switchgrass after 72h regular saccharification is 2.4g/L whereas the cumulative yield after repeated extraction is 2.3g/L.

Xylose yield from 5% switchgrass after 72h regular saccharification is 2.7g/L whereas the cumulative yield after repeated extraction is 4.5g/L. Repeated saccharification of saccharified-biomass does not enhance overall sugar yield as compared with one-time enzyme addition at least in the case of acid-pretreated switchgrass. Using 10ml enzyme in one-time addition of crude enzyme to switchgrass yielded far greater sugar when compared with 30ml enzyme addition by way of repeated saccharification. Even if there were marginal increases in sugar yield (as seen in glucose and xylose yield from 5% switchgrass), it is not proportionate to the amount of enzyme used. After saccharification and ethanol fermentation, inhibitors like acetic acid and furfural were not detected by HPLC and our detection limit was 10µg/L. Using 5% switchgrass may be more suited to obtain higher sugar and ethanol yield with higher ethanol conversion efficiency.

The cellulase and xylanase activities were the highest when the growth medium contained 10mM of only Manganese followed by the screening medium (control) containing a suite of

metal ions which resulted in significantly lesser activities. The activities of cellulase and beta-glucosidase were unaffected by sand-filtration compared to centrifugation whereas activities of enzymes responsible for xylose release namely, xylanase and beta-xylosidase from sand filtration were either greater or lesser than centrifuged samples respectively. This was reflected in the subsequent saccharification of switchgrass where the glucose yields were similar between sand-filtered and centrifuged samples whereas the xylose yield in centrifuged sample was greater than that from sand-filtered sample.

There was at least 24% loss in protein content in sand-filtered enzyme, yet this did not affect the cellulase and beta-glucosidase activities, resulting in glucose yields similar to that from centrifugation. There was 1% increase in cellulase activity, 5% reduction in beta-glucosidase activity and 9% reduction in glucose yield in sand-filtered sample compared to centrifuged sample, all of which are not reflected by the 24% loss in protein by sand filtration. Considering no significant loss of glucose yield by sand filtration, and the fact that a glucose-fermenting yeast namely *Saccharomyces cerevisiae* was employed for ethanol fermentation, sand filtration can be effectively employed to separate *Trichoderma* SG2 biomass from crude enzyme and is an economical substitute to centrifugation. Interestingly, there was 17% increase in xylanase activity, 9% reduction in beta-xylosidase activity and 48% decrease in xylose yield in sand-filtered samples compared to centrifuged sample. Xylose yield is probably influenced by bioactive components lost or reduced during sand filtration and is probably reflected in the 24% loss of protein by sand filtration.

Using tangential flow filtration (TFF), the crude SG2 enzyme was concentrated two-, five- and ten-fold with concomitant increase in protein content, and sugar yield from saccharification of switchgrass. Ten-fold-concentrated enzyme was the best as far as sugar yield and protein content was concerned. However, it should be noted that although TFF increased protein content in the samples, there was substantial loss of protein based on mass balance: there was 40% loss, 58% and 56% loss of protein in the two-, five- and ten-fold concentrated enzyme samples.

The most widely followed pretreatment methods involve mild acid or alkali (2% sulfuric acid or 2% sodium hydroxide). Initially, in this project we found that 2% sulfuric acid was ideal for the pretreatment of switchgrass. We developed different combinations of acid and alkali pretreatments (2% sulfuric acid, 2% sodium hydroxide), very mild acid (0.5% sulfuric acid) and concentrated acid pretreatments (85% phosphoric acid followed by alcohol or acetone). The 85% phosphoric acid followed by acetone resulted in 5.4g/L glucose and 85% phosphoric acid followed by acetone resulted in 4.6 g/L glucose. However, very low concentration of acid and alkali ie., 2% sodium hydroxide followed by 2% sulfuric acid yielded 4.7g/L glucose and the highest xylose yield of 1.5g/L. It was therefore concluded that very low concentration of alkali followed by very low concentration of acid results in high sugar yield and comparatively, the 85% phosphoric acid treatment gives only 15% higher sugar yield. The use of 1L of 85% phosphoric acid and nearly 5L of acetone for pretreatment of 10% switchgrass seems excessive compared to 1L 2% sodium hydroxide and 1L 2% sulfuric acid for pretreatment of 10% switchgrass for only 15% greater sugar yield. It can be concluded that 2% alkali followed by 2% acid is ideal from economical stand point and from the perspective of environmental safety.

Manganese was validated to improve cellulase and xylanase production in *Trichoderma* sp SG2. Addition of Ba, Ca, Co, Fe, K, Mn and Zn to enzyme assay mixture displayed stimulation of enzyme activity compared to assays without addition of metal ions. Results

indicate that metal ions can be added to biomass saccharification reaction mixture to improve sugar yield. Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures revealed that over 50% of the initial anion nutrients remained detectable in cell-free culture supernatant and suggested that half of the mineral nutrients was sufficient for production of cellulolytic and xylanolytic enzymes of *Trichoderma* sp. SG2. Enzyme production with 50% mineral solution revealed that there was no decrease of enzyme activity in cultures.

Ba, Ca and Mn substantially promoted biomass hydrolysis at orders of magnitude ranging from 1.5 to 1.9 times at 1-5 mM. The highest promotion of biomass hydrolysis (1.93 times) was observed with 1mM Mn followed by Ca which displayed 1.89 times increase in biomass hydrolysis at 5 mM. Synergistic effect in promotion of biomass hydrolysis was not observed when the three metal ions were examined in combinations. Dilution of the enzymes before assay revealed that more enzymes are present in the cell-free culture supernatant than required to contact with/and convert the substrate to saccharides. Overall, results suggest potential application of *Trichoderma* SG2 for production of saccharides from biomass with Ba, Ca or Mn supplementation. Interestingly, *Trichoderma* SG2 co-produces cellulolytic-xylanolytic-amylolytic enzymes. This is an indication that starch-rich carbohydrate wastes can be added as adjuncts for higher yield of fermentable saccharides.

Metal ion promotion of biomass saccharification by cellulolytic and xylanolytic enzymes of *Trichoderma* species SG2 was validated by HPLC analysis of amounts of glucose and xylose produced during switchgrass biomass hydrolysis in the presence of barium, calcium and manganese. Addition of Ba, Ca and Mn to biomass saccharification reaction mixture improved glucose and xylose yield compared to the control. Overall, 5mM Ba, Ca and Mn were the most effective for glucose release. Similar results were observed with Ca and Mn for xylose release from switchgrass biomass. Improvement of glucose and xylose release by the three metal ions confirmed the results which indicated that the metal ions promoted cellulolytic and xylanolytic enzyme activities as well as total reducing sugar release.

Biomass saccharification by SG2 enzymes was directly related to temperature between 20^{o}C and 50^{o}C with the maximum yield recorded at 50^{o}C. Sugar yield decreased between 50^{o}C and 60^{o}C. However, stability studies showed that the cellulolytic-xylanolytic enzymes of SG2 were generally less stable above 40^{o}C after 24 hours. This suggested that lower temperature (30^{o}C to 35^{o}C) was more suitable for simultaneous saccharification and fermentation which requires extended incubation time beyond 24 hours. Switchgrass biomass saccharification was pH dependent and maximum release of sugar was observed at pH 5.0-5.5. Addition of starch to lignocellulosic saccharification reaction mixture displayed an “additive effect” in that total reducing sugar yield increased substantially compared to the saccharification of switchgrass or starch, alone. Total sugar obtained from mixed switchgrass and starch hydrolysis was close to the sum of the amounts obtained when the substrates were hydrolyzed singly.

Comparative evaluation of organic nitrogen sources proflo, yeast extract and peptone revealed that a combination of 0.5 g/L yeast extract and 1.5 g/L proflo (YPR) was generally more effective for production of cellulase and β-glucosidase. However, the results are comparable to the previously optimized enzyme production medium in which yeast extract and peptone (YEP) were the organic nitrogen sources. The advantage of YPR is that proflo is a cheap organic nitrogen source; which is a major economic advantage in scale-up fermentation. Proflo compared to yeast extract and peptone proved to be an effective nutrient for enzyme

production. However, it can alter fermentation medium pH and will require pH adjustment with progress of fermentation or high strength buffer to keep the pH stable.

Studies indicated that whole broth simultaneous saccharification and fermentation at 30^{o}C and 35^{o}C compared with cell free enzyme (filtered broth) simultaneous saccharification and fermentation is potentially more cost-effective for converting biomass to ethanol using *Trichoderma* SG2 enzymes.

A bioreactor system for simultaneous saccharification and fermentation; and separate saccharification and fermentation of biomass to ethanol was developed for scale up and optimization of laboratory results. Three independent 200L scale up runs of the bioreactor system for enzyme production did not yield the promising results achieved in the flask experiments. Thus further studies are required to optimize and scale up the process. Interestingly, results from flask experiments showed that use of starch-rich waste materials and other carbohydrate wastes in combination with pretreated biomass are required to make scale up of the process economically attractive. A patent, US 9,617,574 B2, was issued for aspects of the project.

RESEARCH ACTIVITIES

PART 1: Research Activities of FY-11 (November 1, 2010 to March 31, 2011).
Written by: Benedict Okeke and Rosine Hall

Introduction

The collection of composite soil and biomass samples was undertaken in order to seek and culture novel fungal and yeast isolates. Thus, potential high species diversity and relatively high levels of decomposing cellulosic material at the surface were deemed to be important criteria in selecting sample locations. Preliminary research indicated that undisturbed forest sites had been found to be relatively rich in microbial diversity (Thoms et al., 2010). In another study, the authors (Degens et al, 2000) found that soils from undisturbed pastures and pine forests had higher catabolic diversity than cropped soils. It also seemed reasonable that sites used for composting such materials, or with substantial amounts of surface litter might give a high probability of finding novel isolates. Finally, we selected a few agricultural sites with visible remaining surface litter, reasoning that these sites might harbor particular microorganisms adept at decomposing agricultural waste. Geographically, Alabama Black Belt counties, as designated in the grant proposal, were chosen. The Black Belt counties are important agricultural areas in Alabama and were assumed to have received high quantities of ligocellulosic agricultural wastes over the years. Natural vegetation includes oak and pine forests and shortgrass prairie. Corn, cotton, and soybeans are important crops in Alabama Black Belt counties.

Site selection

Forested areas were chosen based on the visual appearance of being relatively undisturbed. Specific criteria included the absence of recent cutting or clearing, the absence of evidence of recent fire, a robust litter layer, no nearby structures, and with a sufficiently closed canopy to provide shade. Preference was given to public property; for private property, permission to sample was obtained from the owner. Forested sites were either mixed species forest or pine forests. Agricultural sites (corn, cotton, and soybean fields) were chosen by visually determining that there was substantial organic biomass cover like burrs, husks and hulls covering the soil. Most of these sites had been untilled for 2 harvesting seasons. A few sites containing long-term compost piles were sampled and these included compost from landscaping waste and composite compost piles from manure, grass clippings and crop surplus.

Methods

Sample collection

The procedure for collecting soil and surface biomass (SSB) was as follows. A small area approximately 100 cm^2 was cleared of all large loose debris, such as logs and limbs. The top litter layer, including dead leaves, small twigs, and decaying organic matter (O layer) was collected by scraping down to the soil surface across the entire area and was placed into a sterile plastic bag. Then, 4-6 shallow soil core samples were taken down to 10 cm of depth. These were composited into the same bag. Ancillary data were also taken including the latitude and longitude (by GPS) and a general description of the sample location. In the lab, samples were promptly refrigerated. Upon time of usage for inoculation of enrichment medium they were thoroughly mixed.

Enrichment of lignocellulose degrading fungi

The enrichment basal medium comprised of the following (in g/L): KH_2PO_4, 2.0g; $(NH_4)_2SO_4$, 1.2g; $MgSO_4 .7H_2O$, 0. 5g; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.003g; yeast extract, 0.2g, tween 80, 0.5g (Okeke 2010 – unpublished) and 2 mL trace elements solution (Focht, 1994). Fifty milliliters of isolation medium, 0.25 g sawdust, and 0.25 g shredded paper were placed into 250 mL flasks, and autoclaved at 121°C for 20 minutes. After allowing the medium to cool to room temperature, 2.5 g of each composited SSB was placed into the sterilized medium. Cultures were incubated in an orbital shaker at 30°C for 3 days. After 3 days, 2.5 mL of each culture was transferred into a fresh isolation medium and further incubated for approximately 3.5 days. All enrichment cultures were set up in duplicates.

Isolation of cellulolytic and xylanolytic monocultures from enrichment cultures

The enrichment culture was diluted serially from 10^{-1} to 10^{-5} in saline (0.9 % NaCl). Diluted enrichment samples (10^{-2} to 10^{-5}) were plated on the enrichment basal medium to which sawdust (4 g/L), carboxymethyl-cellulose (1 g/L), chloroamphenicol, 75 mg/L and agar (20 g/L) were added. Cultures were incubated at 30^oC for 7 days. Discrete fungal colonies were plated on potato dextrose agar and incubated at 30^oC for 3 days. Cultures were preserved at 4^oC until assessed for production of cellulase and xylanase.

Screening of fungal isolates for production of cellulolytic and hemicellulolytic enzymes

Thirty-two putative cellulolytic and xylanolytic fungal isolates were screened for enzyme production in liquid submerged culture. The medium comprised (per liter) of KH_2PO_4, 2.0g; $(NH_4)_2SO_4$, 1.2g; $MgSO_4 .7H_2O$, 0. 5g; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.003g and 2 ml of trace elements solution (Focht, 1994). In independent parallel duplicate experiments, sterile 50 mL of the enrichment medium in 250 mL was inoculated with two 1.25 cm agar blocks obtained from potato dextrose agar plate cultures of each isolate incubated at 30^oC for 3-5 days. Cultures were incubated with orbital shaking (200 rpm) at 30^oC for 7 days. Fungal biomass was removed from cultures by centrifugation (5,000 rpm, 10 min) and the supernatant was assessed for filter paper cellulase and xylanase activities. Ten fungi selected based on cellulase activity of non-concentrated culture supernatant were further assessed for β-glucosidase and β-xylosidase activities.

Comparative evaluation of two laboratory strains of cellulolytic and xylanolytic fungi and *Trichoderma reesei* RUTC30

The hypercellulolytic mutant Hypocrea jecorina (Trichoderma reesei RUTC30) is the most frequently used strain for cellulase fermentations (Seidl et al. 2008). RUT C30 was purchased from ATCC. Two laboratory strains of cellulolytic and xylanolytic fungi Hypocrea (Trichoderma) species SG2 and SG4 previously isolated from composite soil and decaying biomass in the laboratory of Benedict Okeke were compared with a commercial strain, Rut-C30. Media and culture conditions were as described under screening of fungal isolates for production of cellulolytic and hemicellulolytic enzymes. Cultures were incubated for 12 days. Samples were taken every 3 days for analysis of cellulase, xylanase, β-glucosidase and β-xylosidase. Activities of enzymes were measured in culture supernatants (5000 rpm, 10 min).

Analysis of cellulase, xylanase, β-glucosidase, and β-xylosidase activities

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2011). For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2005) and cooled on ice before measuring absorbance at λ_{405}. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside. One unit of enzyme activity was 1 µmol of product released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

Screening of composite surface soil and biomass samples for xylose fermenting yeast

The soil biomass samples were also used for enrichment of xylose fermenting yeasts. This task of selecting novel xylose fermenting yeast is ongoing. More samples will be collected from orchards in Alabama.

Results

Sites for isolation of lignocellulose degrading fungi

Site description and GPS data are presented in Table 1. Sample locations are shown in Fig. 1).

Cellulolytic and xylanolytic enzyme activities

Thirty-two potential cellulolytic and xylanolytic fungal monocultures were isolated from the sites and screened for enzyme production in liquid submerged culture (Fig. 2). Isolate FS23A and FS22 produced the highest cellulolytic activity, 5.03±0.31 and 4.92±0.56 µmole/mL, respectively. Xylanolytic activity was also substantial in both isolates, 26.91±0.2.35 and 29.74±5.91 µmole/mL, respectively. Nonetheless, isolates F25A and FS23 displayed the highest xylanase activities, 31.83±0.1.60 and 30.98±2.37 µmole/mL, respectively.

β-glucosidase and β-xylosidase activities

Based on cellulolytic activity of isolates, ten isolates were selected and assayed for production of β-glucosidase and β-xylosidase activities. High levels of β-glucosidase, 64.20±13.47, 58.12±15 and 53.35±1.38 were detected in cultures of FS18, FS22 and FS25, respectively. β-xylosidase activity was highest in cultures of isolates FS5A and FS12, 55.59±5.62 and 56.30±22.8, respectively.

Comparative production of cellulolytic and xylanolytic enzymes by strains SG4, SG2 and RUT-C30

Fig. 4 presents the time course of production of cellulolytic and xylanolytic enzymes by strains SG4, SG2 and RUT-C30. Strains SG4 and SG2 displayed cellulase production peak on day 3 and cellulase production was relatively stable thereafter. Cellulolytic activity in cultures of RUT-C30 increased with incubation time until day 9. The highest cellulase activity was observed in culture of RUT-C30, 32.45±0.59 and 29.42±6.1 on day 9 and 12, respectively. Cellulase production by SG2 and SG4 were also high, however, RUT-C30 was a more efficient cellulase producer. The three fungal strains produced similar levels of xylanase (Fig. 4). Strains SG4 and SG2

produced significantly higher β-glucosidase activity than the commercial strain RUT-C30. In cultures of SG4 and SG2, β-glucosidase increased with incubation until day 9 and was stable thereafter. Strains SG4 and SG2 also produced more β-xylosidase than RUT-C30 during the 12 days of incubation.

Discussion

Substantial levels of cellulolytic and xylanolytic enzymes were observed in cultures of the fungal isolates. Two laboratory strains, SG2 and SG4, previously isolated from soil beneath decaying switch grass produced higher levels of cellulase and xylanase compared to the thirty-two new isolates. However, some of the new isolates appear to produce more β-glucosidase and β-xylosidase. Overall, strain RUT-C30 produced the highest cellulase activity. This was not surprising because strain RUT-C30 is a hypercellulolytic mutant frequently used for cellulase production (Seidl et al. 2008). Cellulolytic activity of strains SG4 and SG2 are considerably high since they are wild-type strains that have not been subjected to strain improvement. SG4 and SG2 produced as much as half of the cellulolytic activity of the hypercellulolytic mutant strain RUT-30. Synergistic interaction of cellulolytic-xyanolytic enzymes is required for efficient conversion of lignocelluloses to sugars and subsequent fermentation to ethanol. Interestingly, this study revealed diverse cellullolytic-xylanolytic potential among the different isolates evaluated. Such factors as incomplete synergistic enzymes, end-product inhibition (Kumar and Wyman, 2008), enzyme inactivation (Kumar and Wyman, 2009), recalcitrance due to highly ordered structure and crystallinity of cellulose (Kuo and Lee, 2009) and the heterogeneous nature of a variety of plant fiber (Colin et al. 2005), necessitate use of enzyme mixtures for efficient saccharification of lignocelluloses.

β-glucosidase and β-xylosidases are extremely important terminal enzymes involved in lignocellulose bioprocessing to fermentable sugars, glucose and xylose, from non-fermentable intermediates, cellobiose and cello-oligosaccharides (Harnpicharnchai et al 2009), and xylobiose and xylo-oligosaccharides (Yan et al. 2008), respectively. Strains of *Trichoderma* species are the most common cellulase producing microorganism employed industrially (Chen et al 2010). However, they lack high levels of β-glucosidases required for efficient hydrolysis of cellobiose (Juhász et al. 2005). Consequently commercial cellulose preparations are supplemented with β-glucosidases to increase the rate of cellulose hydrolysis (Barnett et 1991). High levels of β-glucosidases will limit the inhibitory effect of cellobiose on endo- and exo-cellulases (Wen et al. 2005, Howell and Stuck, 1975). Interestingly, substantial levels of β-glucosidase was produced by SG4 and SG2 which make them candidate cultures for production of complete mixture of cellulolytic and xylanolytic enzymes for lignocellulose bioprocessing. Moreover the new isolates appear to produce significant amounts of β-xylosidases.

In summary, we isolated monocultures of several cellulolytic-xylanolytic fungi that can produce an array of cellulolytic and xylanolytic enzymes. Selected cultures can be used for co-production of cellulolytic-xylanolytic enzymes as well as simultaneous saccharification and fermentation to ethanol. This however, requires optimization of culture conditions to maximize enzyme production by the isolates.

Table 1: Sampled site description and geographical information

Sample_ID	Date	N1	N2	N3	N4	E1	E2	E3	E4	Latitude	Longitude	Comments
SBD-1	2/9/2011	N	32	22	17.9	W	86	11	8.6	32.37163889	-86.18572222	Hardwoods
SBD-2	2/9/2011	N	32	24	50.8	W	86	13	30.8	32.41411111	-86.22522222	Hardwoods
SBD-3	2/9/2011	N	32	27	4.4	W	86	13	11.2	32.45122222	-86.21977778	compost pile
SBD-4	2/9/2011	N	32	24	44.4	W	86	13	7.9	32.41233333	-86.21886111	Pine forest
SBD-5	2/9/2011	N	32	20	47.2	W	86	5	45	32.34644444	-86.09583333	mixed forest/grassland
SBD-6	2/16/2011	N	32	16	22.5	W	86	33	57.9	32.27291667	-86.56608333	mixed forest/grassland
SBD-7	2/16/2011	N	32	16	12.8	W	86	35	27.5	32.27022222	-86.59097222	Pine forest
SBD-8	2/16/2011	N	32	18	35.8	W	86	51	5.7	32.30994444	-86.85158333	Soy bean field
SBD-9	2/16/2011	N	32	24	4.4	W	86	59	46	32.40122222	-86.99611111	mixed forest/grassland
SBD-10	2/16/2011	N	32	23	20	W	86	59	43.4	32.38888889	-86.99538889	cotton field
SBD-11	2/16/2011	N	32	19	12	W	86	53	53.7	32.32	-86.89825	corn field
SBD-12	2/16/2011	N	32	19	12	W	86	53	53.7	32.32	-86.89825	pine straw
SBD-13	2/23/2011	N	32	15	17	W	86	8	59	32.25472222	-86.14972222	pine/mixed forest
SBD-14	2/23/2011	N	32	9	14	W	86	7	50.1	32.15388889	-86.13058333	mixed forest/clay
SBD-15	2/23/2011	N	31	56	31.7	W	86	1	24.2	31.94213889	-86.02338889	mixed forest
SBD-16	2/23/2011	N	31	43	18.4	W	86	13	15.3	31.72177778	-86.22091667	wetlands/pines above
SBD-17	2/23/2011	N	31	43	10.6	W	86	13	15.4	31.71961111	-86.22094444	muddy/organic rich
SBD-18	2/23/2011	N	31	42	56.2	W	86	8	47.4	31.71561111	-86.1465	corn field
SBD-19	2/23/2011	N	31	48	27.2	W	86	1	55.7	31.80755556	-86.03213889	mixed forest
SBD-20	2/23/2011	N	31	54	56.9	W	86	0	39.9	31.91580556	-86.01108333	mixed forest
SBD-21	2/23/2011	N	32	19	9.3	W	86	9	48.7	32.31925	-86.16352778	Pine forest
SBD-22	3/2/2011	N	32	21	56.6	W	86	3	48.8	32.36572222	-86.06355556	compost pile
SBD-23	3/2/2011	N	32	22	1.6	W	86	4	3.4	32.36711111	-86.06761111	landscape debris pile
SBD-24	3/2/2011	N	32	26	54.5	W	85	38	1.1	32.44847222	-85.63363889	mixed forest
SBD-25	3/2/2011	N	32	26	13.2	W	85	23	13.8	32.437	-85.38716667	mixed forest/low woody vines
SBD-26	3/2/2011	N	32	26	8.7	W	85	35	9.8	32.43575	-85.58605556	mixed forest
SBD-27	3/2/2011	N	32	23	58	W	85	52	23.7	32.39944444	-85.87325	mixed forest
SBD-28	3/2/2011	N	32	23	54.9	W	85	57	48.4	32.39858333	-85.96344444	Pine forest

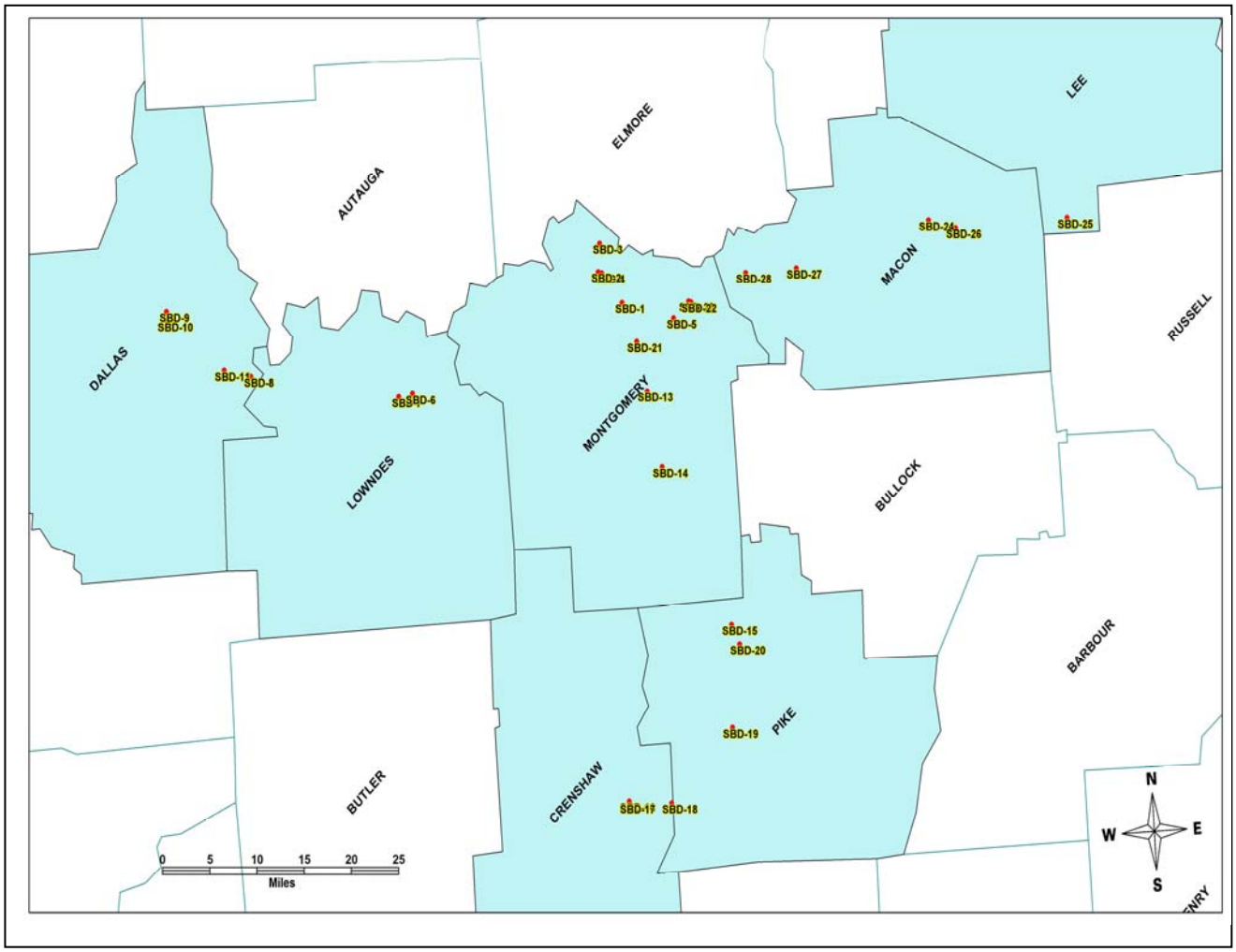

Fig. 1: Map of sites sampled

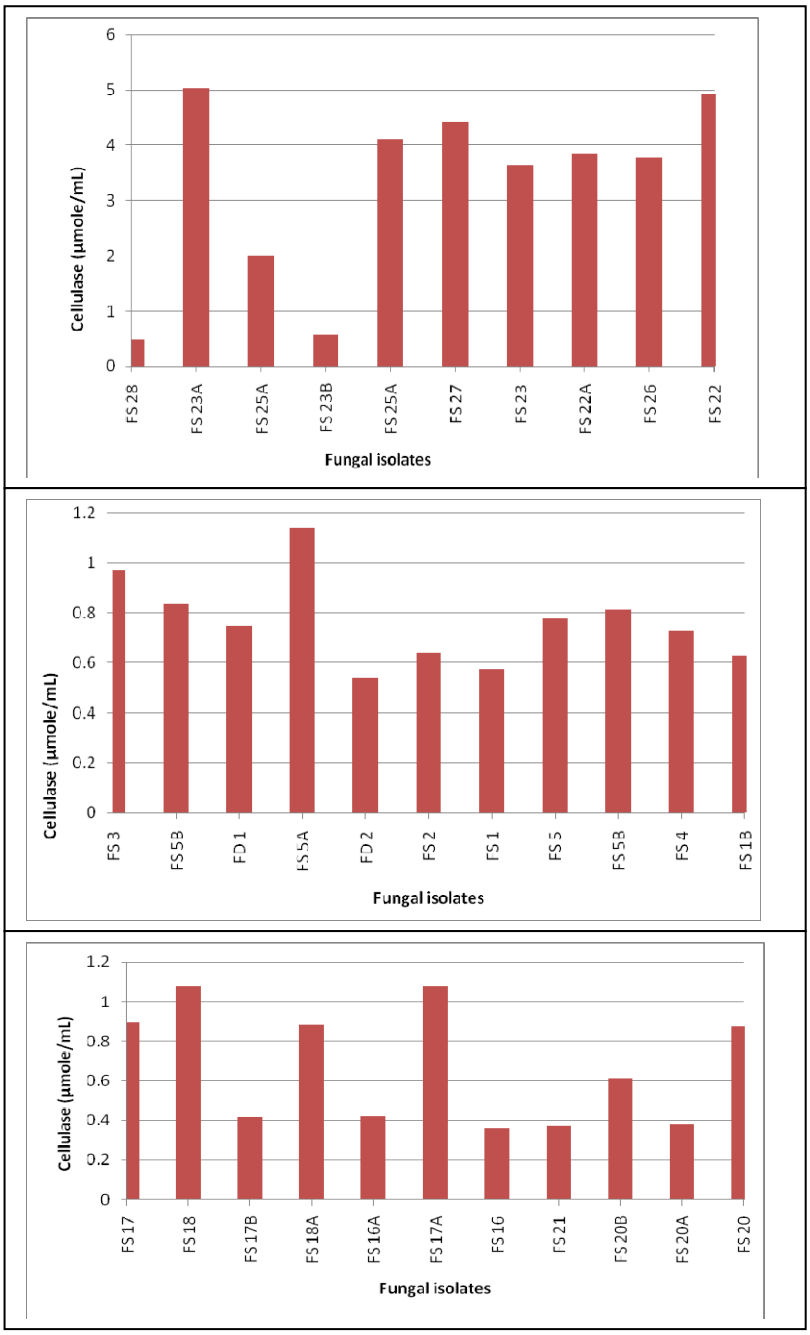

Fig. 2: Cellulase activity of thirty-two fungal isolates. Values are enzyme activities recorded in a 30 min assay.

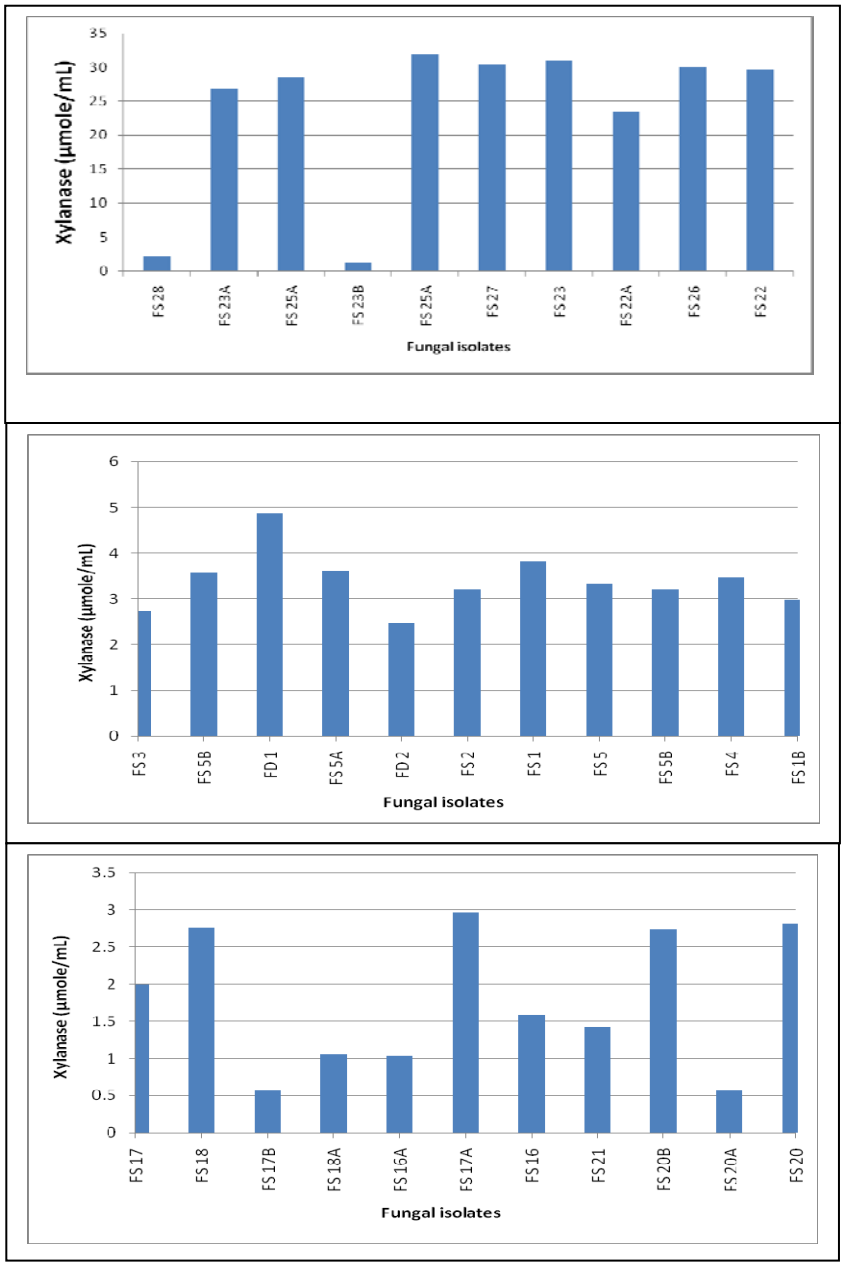

Fig. 3: Xylanase activity of thirty-two fungal isolates. Values are enzyme activities recorded in a 30 min assay.

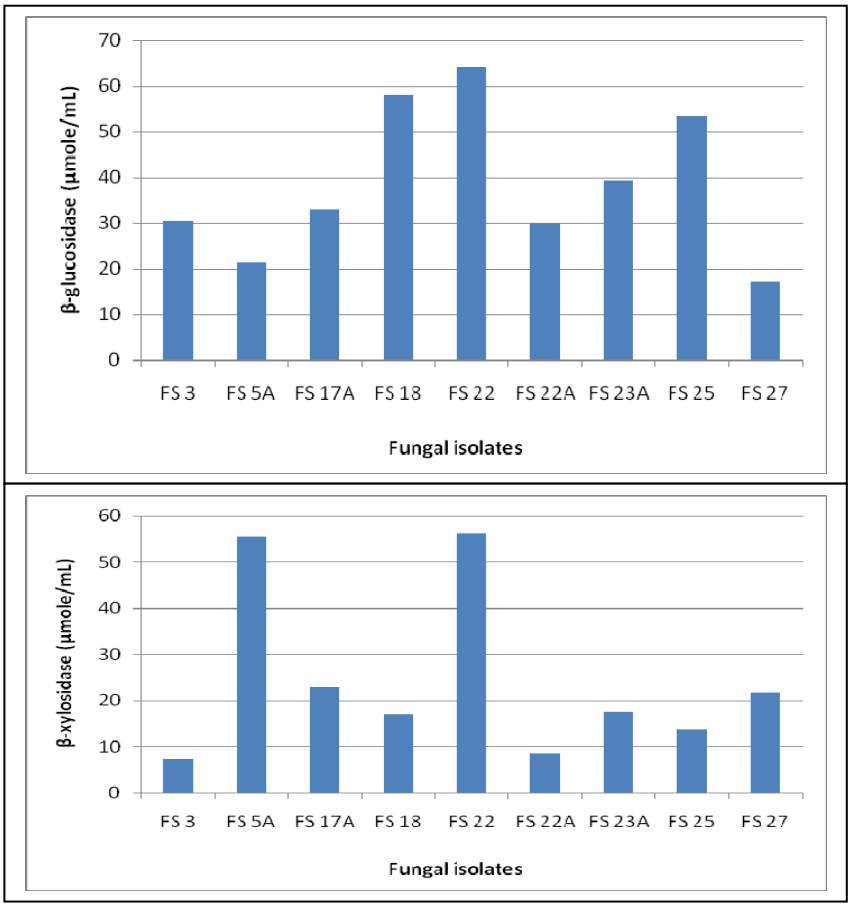

Fig. 4: β-glucosidase and β-xylosidase activities in culture filtrate of selected fungal isolates. Values are enzyme activities recorded in a 30 min assay.

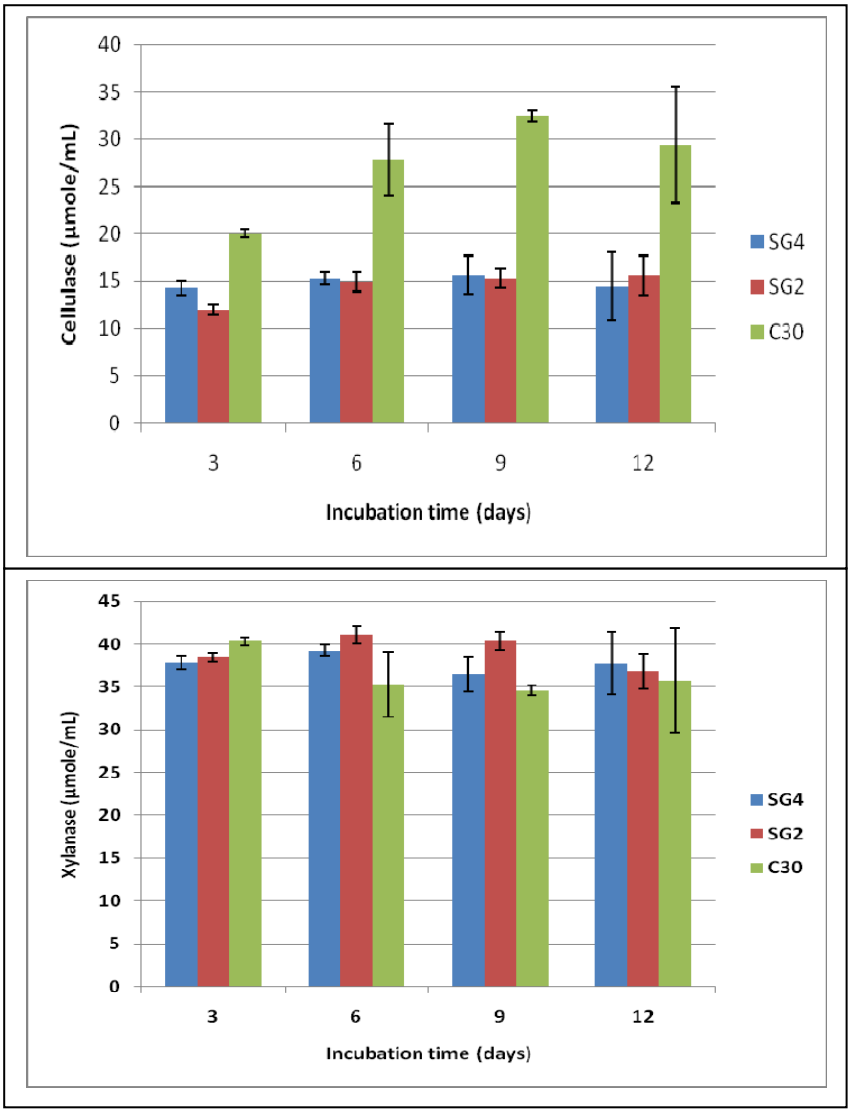

Fig.5: Time course of cellulase and xylanase production by strains SG4, SG2 and RUT-C30 (C30).

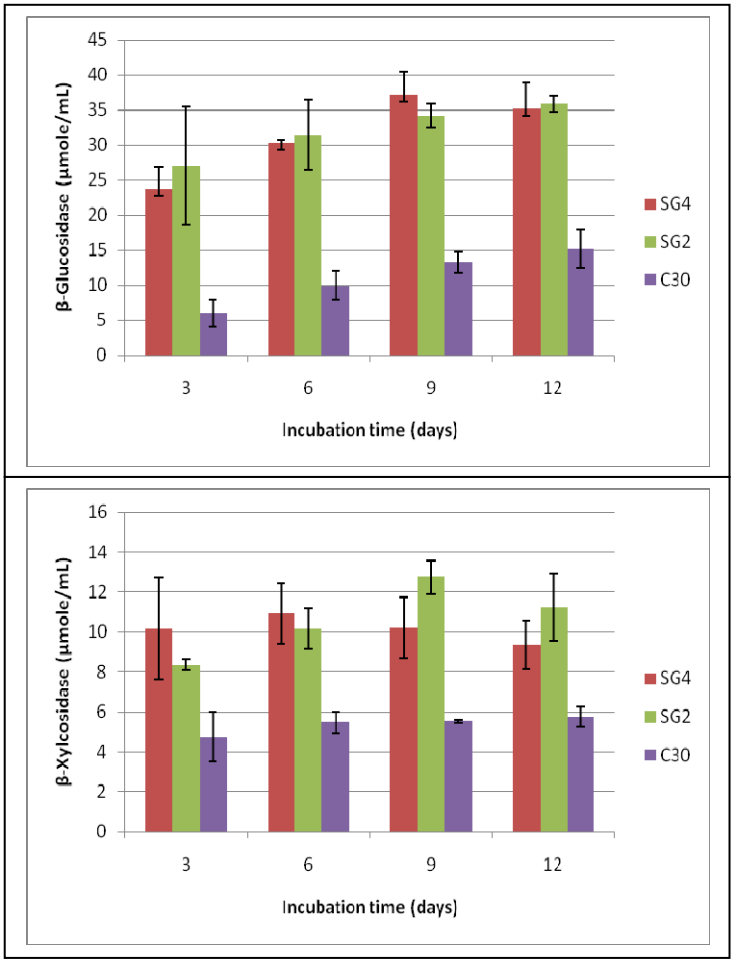

Fig.6: Time course of β-glucosidase and β-xylosidase production by strains SG4, SG2 and RUT-C30 (C30).

Presentations

1. Andrew Prescott, Yasi Deravi, Shanticia Peaks, Jamie Bishop, Leah Sawyer, Rosine Hall and Benedict Okeke (2011). Scereening of xylose-utilizing yeast isolates for ethanol production from biomass (Poster). Auburn Montgomery Undergraduate Research Symposium.

2. Yasi Deravi, Andrew Prescott, Shanticia Peaks, Jamie Bishop, Leah Sawyer, Rosine Hall and Benedict Okeke (2011). Isolation of plant fiber-degrading fungi for conversion of lignocelluloses to sugars. Auburn Montgomery Undergraduate Research Symposium.

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PART 2: Research Activities of FY-11 (April 1, 2011 to June 30, 2011).

Written by: Benedict Okeke, Ananda Nanjundaswamy and Rosine Hall

Introduction

Based on screening of several cellulollytic and xylanolytic fungal isolates and comparison to a commercial strain Rut-C30, a newly isolated *Trichoderma* sp. SG-2 was selected for optimization of culture parameters for enhanced production of cellulase, xylanase, β-glucosidase and β-xylosidase.

Materials and methods

Preservation of cultures

Tricoderma sp. SG-2 and other isolates were preserved on potato dextrose agar (PDA) and stored at 4ºC until further use.

Inoculum generation

Either agar discs or mycelia pellets/granules were employed. Cultures were sub-cultured on PDA and incubated at 30ºC for 72h. Well grown plates were stored at 4ºC and when required, agar discs were bored using sterile metallic borer for inoculation. Two agar discs carrying near-uniform confluent mycelia were used to inoculate each flask. For preparation of mycelial pellets/granules, a loopful of SG2 was inoculated into control medium containing 10g/L (see composition below) all-purpose flour replacing 5g of paper powder and 5g of switchgrass and incubated for 48h at 30ºC at 200 rpm. About 10% of inoculum was used to inoculate media.

Media composition

Submerged fermentation (SmF)

Control or Screening medium: A liter of the medium contained 5.0g of powdered waste paper, 5.0g of pulverized switch grass, 1.0g of peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral elements solution (Fotch, 1994).

Substrate evaluation media: Eight different substrates namely sawdust, switchgrass, peanut hull, paper powder, corn cob, molasses, glucose and xylose were evaluated for enzyme production. Except for glucose, xylose and molasses which were used at 2% concentration, the remaining substrates were used at 1% concentration by replacing the sawdust and switchgrass in the screening medium. Glucose and xylose media were used for reference only.

Media for evaluation of nitrogen sources: Six different nitrogen sources namely, ammonium citrate, ammonium sulfate, urea, corn steep liquor, peptone and yeast extract were evaluated. Screening medium composition was modified to contain (g/l) 2g KH_2PO_4, 10.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, 0.5g Tween 80, 2ml Fotch mineral element solution, 5g of shredded waste paper, 5.0g of pulverized switch grass and amended with the respective nitrogen source. Since Brown et al (1998) recommended the use of 0.8g/l equivalent of nitrogen and was employed by Chavez et al (2004) for amylase production in *Trichoderma*, the different nitrogen

sources in this study were incorporated accordingly (6.5g/l ammonium citrate, 3.8g/l ammonium sulfate, 1.7g/l urea, corn steep liquor 23.5g/l, 5.7g/l peptone or 7.6g/l yeast extract).

Media for evaluation of surfactants: Control medium was supplemented with 0.5g of five surfactants namely TritonX-100, Tween-20, 40, 60 or 80.

Solid state fermentation (SSF)

Nine substrates namely bermuda grass, corn cob, peanut hull, paper powder, sawdust, soy hull, switch grass, wheat bran and mixture of all substrates were evaluated. About 100g of the substrate was mixed with 30g of water and sterilized by autoclaving at 121ºC for 30 min and upon cooling to room temperature was inoculated with 140 ml of 1:10 inoculum to obtain a final moisture content of 62%. After mixing the contents thoroughly, 5g portions of the mixture were dispensed into sterile disposable mini-plates (50mm×10mm). In the case of mixture of substrate, 12.5g of each of the eight substrates were mixed together using blender. Each treatment received 12 plates and three plates (replicates) were harvested completely on days 3, 5, 7 and 9 of fermentation. A known quantity of the sample was extracted with demineralized water, centrifuged at 5000 rpm for 5 min and supernatant was used for enzyme assay.

Fermentation conditions

For optimization of temperature, pH, nitrogen source and surfactant in shake flasks, screening medium was used. Comparison of enzyme activities in different fungal isolates and RutC30 were also conducted using control medium. Fermentation was carried out in 250 ml flasks and each flask contained 50 ml of respective media. Flasks were sterilized at 121ºC for 20 min. Upon cooling to room temperature, flasks were inoculated aseptically with two 3 mm fungal agar discs. Each treatment received three replicates. Flasks were incubated for 4 days at 30ºC (except when specified), pH 6.2 (except when specified) and 200 rpm. The end of fermentation samples were centrifuged at 5000 rpm for 10 min and supernatant collected in a clean tube for enzyme assay.

For temperature optimization, flasks were incubated at 25, 30, 35, 40 or 50ºC in water bath shaker. For pH optimization, flasks were maintained at pH 4, 5, 6, 7 or 8. Medium pH was adjusted using 2.5M HCl or 5M NaOH.

Enzyme assay

End of fermentation samples were analyzed for cellulase, xylanase, β-glucosidase and β-xylosidase. Method described in Saha et al (2005) was followed. Briefly, filter paper discs were used for cellulase assay. About 10 discs were placed in glass test tubes and 0.5 ml of 100 mM sodium acetate buffer at pH 5.0 was added (Okeke and Lu, 2011). About 0.5 ml of end of fermentation sample (crude enzyme extract) was added and tubes were incubated at 50ºC for 30 min. Enzyme reaction was stopped with I ml DNS reagent and 0.5 ml of 40% sodium potassium tartarate was added, sample mixture was placed in a boiling water bath for 10 min and cooled to room temperature. Using a spectrophotometer, optical density was read at 575nm, and reducing sugar released was calculated using standard glucose curve. The same procedure was followed for xylanase assay except that the substrate was oats xylan.

For β-glucosidase and β-xylosidase, substrates *p*-nitrophenyl β-D-glucoside and *p*-nitrophenyl β-D-xyloside were respectively used. In brief, approximately 800 µl of 100 mM acetate buffer was added to 100 ml of 40 mM of substrate. About 100 µl of crude enzyme extract was added, incubated at 50ºC for 30 min and immediately transferred onto ice cold water bath. The enzyme reaction was stopped using 1ml of chilled 0.5 M Na_2CO_3. Sample OD was read at 405 nm and enzyme activity expressed as micro moles of *p*-nitrophenol released per milliliter of enzyme extract was calculated by using standard curve.

For optimization of assay temperature, the enzyme samples were incubated at different temperatures, namely 30, 40, 50, 60, 70 and 80ºC in a water bath. Stability of enzyme activity at optimum assay temperature was evaluated by incubating the samples at optimum assay temperature in a water bath and assayed at times 0h, 0.5h, 1.0h, 2.0h, 4.0h and 24h. Effect of assay pH on enzyme activity was evaluated by using acetate buffer with pH ranging from 4 to 7.

Enzyme activities were expressed as Units/ml/30 min in SmF and U/g dry weight/min in SSF.

Response surface methodology (RSM)

RSM was employed to study the effect of the proportion of four ingredients of control (screening) medium, namely paper powder, switchgrass, yeast extract and peptone on enzyme activity (Table 2). Design expert 7.1.6 (Stat Ease Inc., Minneapolis,Mn,USA) was used in generating design matrix and analysis of results.

Statistics

Statistical analyses were conducted using SAS (version 9.1.3). Proc ANOVA was used to determine differences between treatments and Tukey pair-wise correction was used. Significance was set at P=0.05.

Results

Optimization of *Trichoderma* SG2 growth conditions for enzyme production in SmF

Substrate

The cellulase activities were not statistically different among the seven different substrates (Fig.1). However, the highest cellulase activity was seen in molasses (3.46±0.75), followed by switchgrass (2.26±0.43). The xylanase activities were significantly different and the highest activity was seen in paper powder (22.52±2.37) followed by switchgrass (17.35±1.35). The β-glucosidase and β-xylosidase activities were also significantly different and the highest β-glucosidase and β-xylosidase activities were seen in the screening (control) medium (21.19±1.35 and 7.63±0.31 respectively) followed by paper powder (2.97+0.35) for β-glucosidase and switchgrass (0.68±0.02) in β-xylosidase, respectively. Based on the enzyme activities and the statistical analyses, it seems that control (screening medium) containing mixture of paper powder and switchgrass is ideally suited followed by switchgrass for enzyme production in submerged fermentation.

Nitrogen source

The activities of all four enzymes were significantly different with the use of various nitrogen sources (Fig.2). The nitrogen source in the control (screening) medium, namely 1.0g peptone, 0.5g yeast extract and 1.2g ammonium sulfate resulted in the highest activities for all four enzymes (Fig. 2): 9.85±0.55 cellulase, 38.91±0.31 xylanase, 21.19±1.35 β-glucosidase and 7.63±0.31 β-xylosidase respectively. However, among the six selected nitrogen sources, mineral sources like ammonium sulfate followed by ammonium citrate resulted in the highest enzyme activities, and corn steep liquor resulted in the highest enzyme activities among the organic sources. The nitrogen sources present in control (screening) medium, namely peptone, yeast extract and ammonium sulfate are optimal for production of all four enzymes.

Surfactant

The activities of all four enzymes were not significantly different when control medium was supplemented with different surfactants (Fig.3). However, Tween-20 resulted in highest cellulase (4.1±0.31) and xylanase activities (36.38±0.95), Tween-60 resulted in highest β-glucosidase (9.56±0.23) and Tween-40 in β-xylosidase (4.7 ±0.13). Based on enzyme activities, Tween-20 seems to be the ideal surfactant.

Temperature

The activities of all four enzymes were significantly different when *Trichoderma* SG2 was grown at different incubation temperatures (Fig.4). The highest cellulase activity (4.86±0.55) was at 25ºC whereas 35ºC supported the highest activities of xylanase (21.94±0.72), β-glucosidase (6.29±0.35) and β-xylosidase (3.69±0.16). Since the activities of all enzymes except xylanase at 30ºC were not significantly different from the respective highest enzyme activities, 30ºC was found to be optimal growth temperature for *Trichoderma* SG2.

pH

The pH of growth medium affected the enzyme activities significantly (Fig. 5). pH 7 resulted in the highest cellulase (11.29±0.6) and β xylosidase (2.6±0.38) activities, pH 8 in xylanase (27.44±0.84) and pH 6 in β glucosidase (11.3±0.08). Since pH 7 yielded the highest activities for all enzymes except xylanase, pH 7 was found to be optimal for growth of *Trichoderma* SG2.

Response surface methodology

A response surface study consisting of *IV-optimal* design with 25 runs without blocking and quadratic design model was carried out to study the effect of the proportion of four ingredients of control (screening) medium, namely paper powder, switchgrass, yeast extract and peptone on enzyme activity (Table 2). Summary of statistical models used for the responses is outlined in Table 3. Design Expert 7.1. 6 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experimental designs, estimate the responses of dependent variables, and generate the contour and/or response surface plots.

Table 4 provides the ANOVA for cellulase activity. The model was significant, with an F value of 4.06. The coefficient estimates and their corresponding P values suggest that only switchgrass and peptone and their interaction are significant.

Main effects of all four ingredients positively influenced the cellulase activity. Fig. 6 represents contour plot for cellulase activity.

Table 5 provides the ANOVA for xylanase activity. The model was significant, with an F value of 3.18. The coefficient estimates and their corresponding P values suggest that only peptone and the interaction of switchgrass and peptone are significant. Main effects of all four ingredients positively influenced the xylanase activity. Fig. 7 represents contour plot for xylanase activity.

Table 6 provides the ANOVA for β glucosidase activity. The model was significant, with an F value of 5.48. The coefficient estimates and their corresponding P values suggest that only peptone, yeast extract, the interaction of switchgrass and peptone and interaction of peptone and yeast extract are significant. Main effects of factors were positive except for yeast extract. Fig. 8 represents contour plot for β- glucosidase activity.

Table 7 provides the ANOVA for β xylosidase activity. The model was significant, with an F value of 5.48. The coefficient estimates and their corresponding P values suggest that all factors except yeast extract, and the interactions of switchgrass and peptone and that of peptone and yeast extract are significant. Main effects of all factors were positive except for peptone. Fig. 9 represents contour plot for β xylosidase activity.

Overall, point prediction from RSM predicts the responses at optimal concentration of ingredients (paper powder 0.62%, switchgrass 0.96%, yeast extract 0.06% and peptone 0.14%).

Effect of assay conditions on enzyme activity

Temperature

Assay temperature significantly influenced the enzyme activities (Fig. 10). The highest cellulase (5.01±0.24) and xylanase (31.7±0.46) activities were seen at assay temperature of 50ºC, highest β-xylosidase (4.34±0.31) activity at 40ºC and β-glucosidase (17.7±0.37) at 70ºC. β-xylosidase activity at 50ºC was not significantly different from the highest activity at 40 ºC. At 80ºC, only β-glucosidase activity was recorded. This study showed that 50ºC is the optimum assay temperature since at least three enzymes exhibited highest activities.

Enzyme stability at optimum assay temperature

Incubation time of enzymes at optimum assay temperature of 50ºC significantly affected the enzyme activities (Fig. 11). The highest cellulase activity (1.66±0.03) was seen after 4h incubation, xylanase (5.54±0.21) and β-glucosidase (7.91±0.89) after 0.5h and β-xylosidase (4.20±0.1) at 0h. However, enzyme activities continued after 24h.

Assay pH

The activities of cellulase, β-glucosidase and β-xylosidase were significantly different at the tested assay pH (Fig. 12). Initial pH 5 resulted in highest cellulase (5.57±0.28),

β-glucosidase (6.29±0.16) and β-xylosidase (8.26±0.54) activities. pH 6 resulted in highest xylanase (31.07±3.01) which was not significantly different from that at pH 5 (30.86±3.16). pH 5 is optimum for enzyme assay.

Enzyme activities from SSF

The enzyme activities on nine different substrates are reported (Fig. 13 and 14). Statistical analyses revealed that the activities varied significantly for some substrates. However, that information is not presented in the graphs as it leads to overcrowding. Cellulase activity was highest in soy hull on day 7 (25.25±0.43; Fig. 13a), xylanase was highest in paper powder on day 7 (143.29±10.6; Fig. 13b), β-glucosidase on soy hull on day 3 (20.06±1.34; Fig. 14a) and β-xylosidase on bermuda grass on day 3 (15.7±4.7; Fig. 14b). Overall, based on statistical analyses and practical feasibility, soy hull fermentation for three days seems ideally suited for enzyme production in SSF.

Comparison of enzyme activities from SG2 and other in-house shortlisted fungi

The activities of all four enzymes of SG2, FSA5 and FSA22 were significantly different (Fig. 15a). FSA22 produced the highest cellulase activity (9.36±0.14) whereas SG2 produced the highest activities of xylanase (36.07±1.5), β-glucosidase (9±1.29) and β-xylosidase (3.69±0.5).

Activities of all enzymes except xylanase were significantly different for SG2, RutC30 and their mixture (Fig.15b). Although RutC30 produced the highest cellulase activity (9.83±0.51), SG2 produced the highest xylanase (43.06±1.32), β-glucosidase (10.41±0.29) and β-xylosidase (4.88±0.08). Overall, SG2 isolate seems to be a better enzyme producer than the commercial mutant strain RutC30.

Table 1: Ingredient variables and their levels tested in optimal design

Factor	Name	Units	Minimum	Mean	Maximum
A	Paper powder	g/50ml	0.1	0.3	0.5
B	Switch Grass	g/50ml	0.1	0.3	0.5
C	Yeast extract	g/50ml	0.01	0.03	0.05
D	Peptone	g/50ml	0.03	0.08	0.13

Table 2: Experimental design matrix and enzyme activities

Run	Factor 1 A:Paper powder g/50ml	Factor 2 B:Switch Grass g/50ml	Factor 3 C:Yeast extract g/50ml	Factor 4 D:Peptone g/50ml	Response 1 Cellulase U/ml	Response 2 Xylanase U/ml	Response 3 β-Glucosidase U/ml	Response 4 β-xylosidase U/ml
1	0.43	0.1	0.05	0.13	2.81	42.4	0.45	1.62
2	0.5	0.35	0.05	0.08	4.04	43.41	15.99	2.74
3	0.5	0.35	0.05	0.08	2.35	40.45	5.45	0.92
4	0.5	0.1	0.05	0.03	1.86	45.51	10.02	4.4
5	0.5	0.5	0.01	0.03	2.56	41.33	11.41	1.38
6	0.31	0.48	0.03	0.07	3.91	40.71	11	5.61
7	0.31	0.48	0.03	0.07	3.41	41.62	11.84	4.59
8	0.5	0.5	0.03	0.13	0.87	32.69	0.53	0.37
9	0.28	0.31	0.01	0.04	4.7	40.3	7.9	5.45
10	0.35	0.1	0.03	0.06	6.4	42	9.98	5.12
11	0.31	0.5	0.05	0.03	4.58	43.81	11.5	6.79
12	0.5	0.1	0.01	0.13	4.42	40.66	15.99	4.37
13	0.1	0.35	0.03	0.03	5.05	42.36	2.15	5.75
14	0.1	0.1	0.02	0.13	4.87	37.42	9.33	4.73
15	0.29	0.36	0.01	0.13	4.32	42.75	6.92	2.16
16	0.47	0.3	0.03	0.03	4.9	40.58	5.05	4.29
17	0.1	0.1	0.01	0.03	3.31	37.44	4.24	3.23
18	0.35	0.1	0.03	0.06	4.58	39.67	9.16	4.56
19	0.1	0.5	0.01	0.07	5.03	39.25	9.33	4.65
20	0.29	0.36	0.01	0.13	3.29	38.67	4.68	2.68
21	0.1	0.5	0.05	0.13	1.17	39.35	0.42	0.45
22	0.1	0.35	0.03	0.03	4.64	45.22	6.7	5.08
23	0.35	0.45	0.05	0.13	0.81	32.46	0.36	0.51
24	0.1	0.1	0.05	0.04	3.3	40.28	8.21	4.74
25	0.12	0.18	0.05	0.13	3.98	39.47	5	0.67

Table 3: Summary of statistical models used for analyses

Response	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1:Cellulase	U/ml	25	Polynomial	0.81	6.40	3.65	1.43	7.88	None	Quadratic
Y2:Xylanase	U/ml	25	Polynomial	32.46	45.51	40.39	3.13	1.40	None	2FI
Y3: β-glucosidase	U/ml	25	Polynomial	0.36	15.99	7.34	4.53	44.18	Natural Log	Quadratic
Y4:β-xylosidase	U/ml	25	Polynomial	0.37	6.79	3.47	1.96	18.48	Natural Log	Quadratic

Table 4 : Cellulase ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	41.5	14	2.96	4.06	**0.0157**
A	4.17	1	4.17	5.71	**0.0379**
B	4.56	1	4.56	6.25	**0.0314**
C	3.24	1	3.24	4.45	0.0611
D	2.52	1	2.52	3.46	0.0925
AB	0.93	1	0.93	1.28	0.284
AC	4.78E-04	1	4.78E-04	6.55E-04	0.9801
AD	0.18	1	0.18	0.25	0.6288
BC	0.46	1	0.46	0.63	0.4458
BD	9.01	1	9.01	12.36	**0.0056**
CD	1.29	1	1.29	1.76	0.2137
A^2	0.42	1	0.42	0.58	0.4641
B^2	2.29	1	2.29	3.14	0.1069
C^2	1.31	1	1.31	1.8	0.209
D^2	3.52	1	3.52	4.82	0.0528
Residual	7.29	10	0.73		
Lack of Fit	3.47	5	0.69	0.91	0.5407
Pure Error	3.82	5	0.76		
Cor Total	48.79	24			

Significant P values are bold-faced

Table 5: Xylanase ANOVA for Response Surface 2FI Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	163.43	10	16.34	3.18	**0.0239**
A	0.2	1	0.2	0.039	0.8455
B	1.68	1	1.68	0.33	0.5763
C	7.94	1	7.94	1.55	0.2341
D	57.6	1	57.6	11.22	**0.0048**
AB	34.65	1	34.65	6.75	**0.021**
AC	0.022	1	0.022	4.27E-03	0.9488
AD	1.22	1	1.22	0.24	0.6332
BC	5.46	1	5.46	1.06	0.3199
BD	23.93	1	23.93	4.66	0.0487
CD	13.74	1	13.74	2.68	0.124
Residual	71.86	14	5.13		
Lack of Fit	51.93	9	5.77	1.45	0.3568
Pure Error	19.93	5	3.99		
Cor Total	235.29	24			

Significant P values are bold-faced

Table 6: β-glucosidase ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	29.07	14	2.08	5.48	**0.0052**
A	2.17E-05	1	2.17E-05	5.72E-05	0.9941
B	0.86	1	0.86	2.27	0.1625
C	2.52	1	2.52	6.65	**0.0274**
D	4.54	1	4.54	11.97	**0.0061**
AB	0.33	1	0.33	0.86	0.3748
AC	0.35	1	0.35	0.93	0.3585
AD	1.78	1	1.78	4.69	0.0557
BC	0.23	1	0.23	0.61	0.4532
BD	2.36	1	2.36	6.22	0.0317
CD	6.25	1	6.25	16.48	**0.0023**
A^2	0.13	1	0.13	0.34	0.5704
B^2	0.17	1	0.17	0.46	0.5128
C^2	0.76	1	0.76	1.99	0.1883
D^2	7.13	1	7.13	18.8	**0.0015**
Residual	3.79	10	0.38		
Lack of Fit	2.48	5	0.5	1.9	0.2491
Pure Error	1.31	5	0.26		
Cor Total	32.86	24			

Significant P values are bold-faced

Table 7: β-xylosidase ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-alue Prob > F
Model	17.94	14	1.28	9.3	**0.0006**
A	1	1	1	7.25	**0.0226**
B	0.99	1	0.99	7.21	**0.0229**
C	0.6	1	0.6	4.37	0.0631
D	5.19	1	5.19	37.69	**0.0001**
AB	0.48	1	0.48	3.51	0.0906
AC	0.15	1	0.15	1.11	0.317
AD	0.018	1	0.018	0.13	0.7248
BC	0.016	1	0.016	0.12	0.7382
BD	1.42	1	1.42	10.31	**0.0093**
CD	2.6	1	2.6	18.84	**0.0015**
A^2	0.62	1	0.62	4.5	0.06
B^2	0.017	1	0.017	0.12	0.7321
C^2	0.065	1	0.065	0.47	0.5064
D^2	0.56	1	0.56	4.06	0.0717
Residual	1.38	10	0.14		
Lack of Fit	0.72	5	0.14	1.1	0.4596
Pure Error	0.66	5	0.13		
Cor Total	19.31	24			

Significant P values are bold-faced

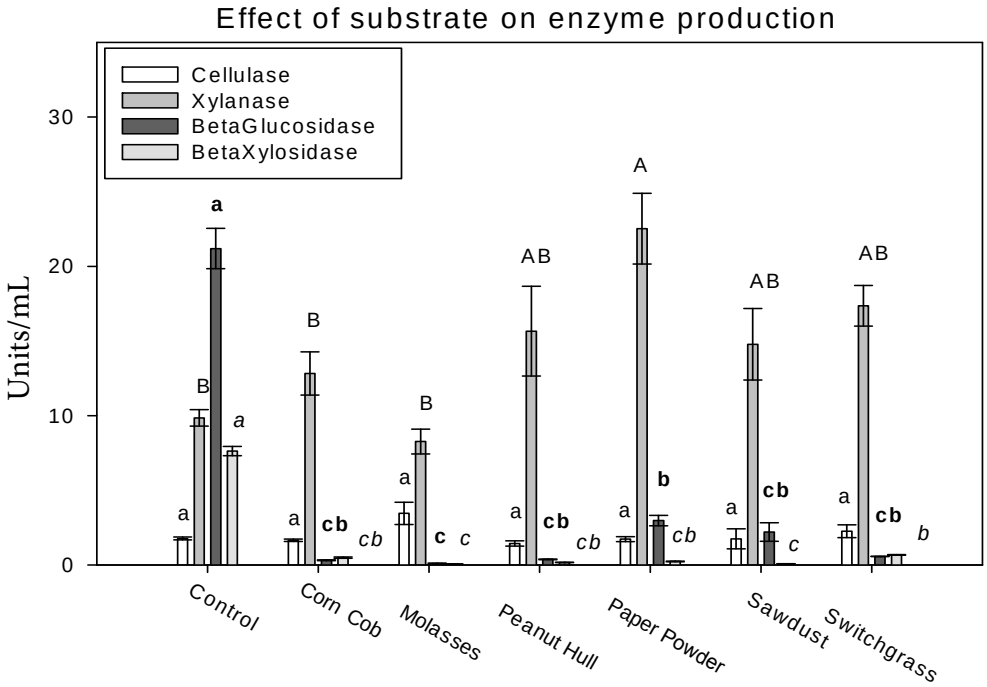

Figure 1. Effect of substrates on enzyme production inbiobased substrates. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

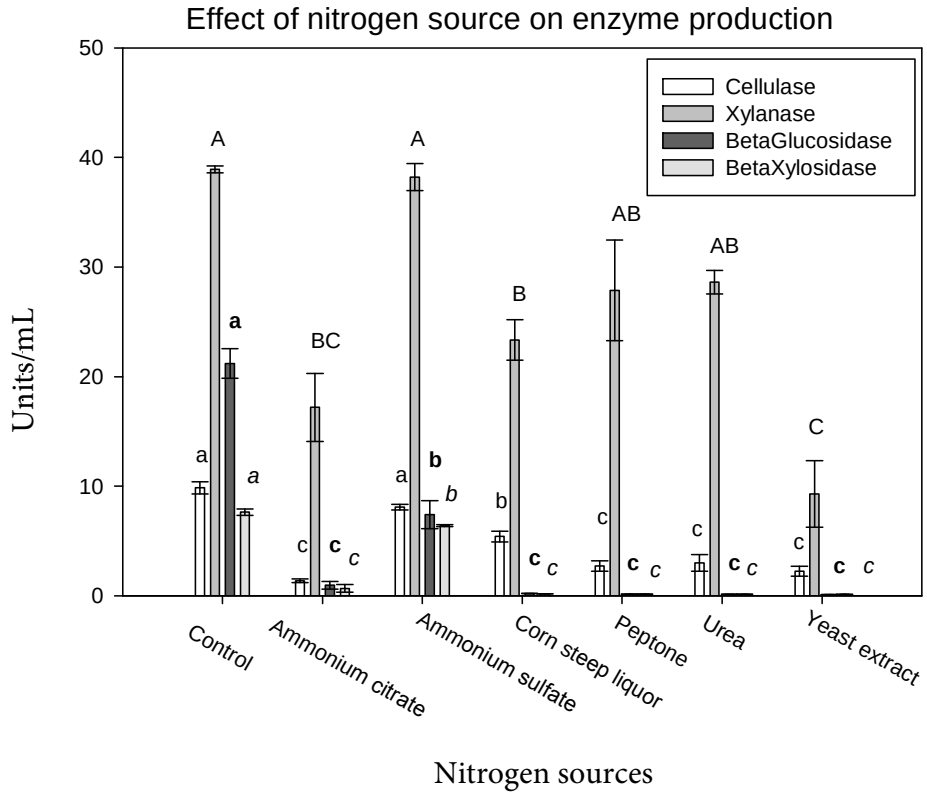

Figure 2: Effect of nitrogen sources on enzyme production. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

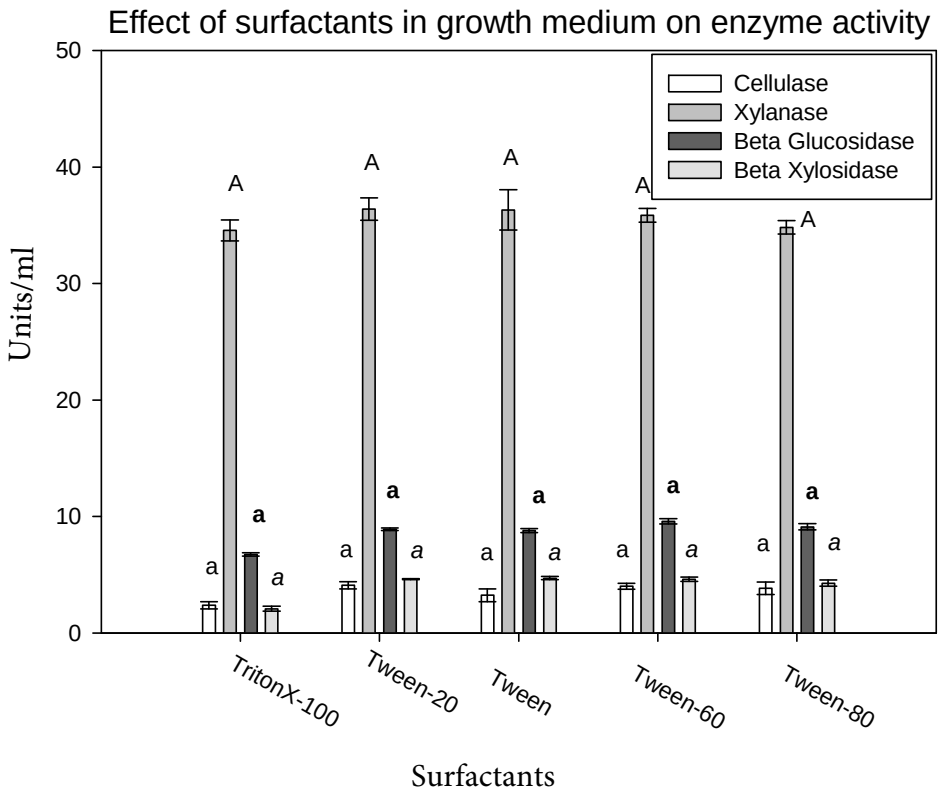

Figure 3: Effect of surfactants on enzyme activity. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

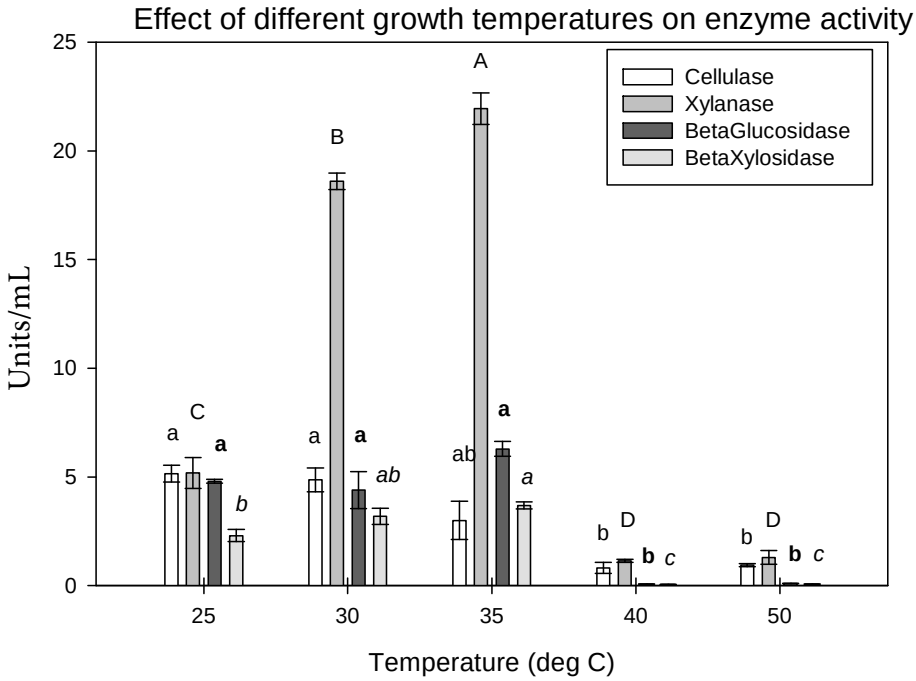

Figure 4: Effect of growth temperatures on enzyme activity. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

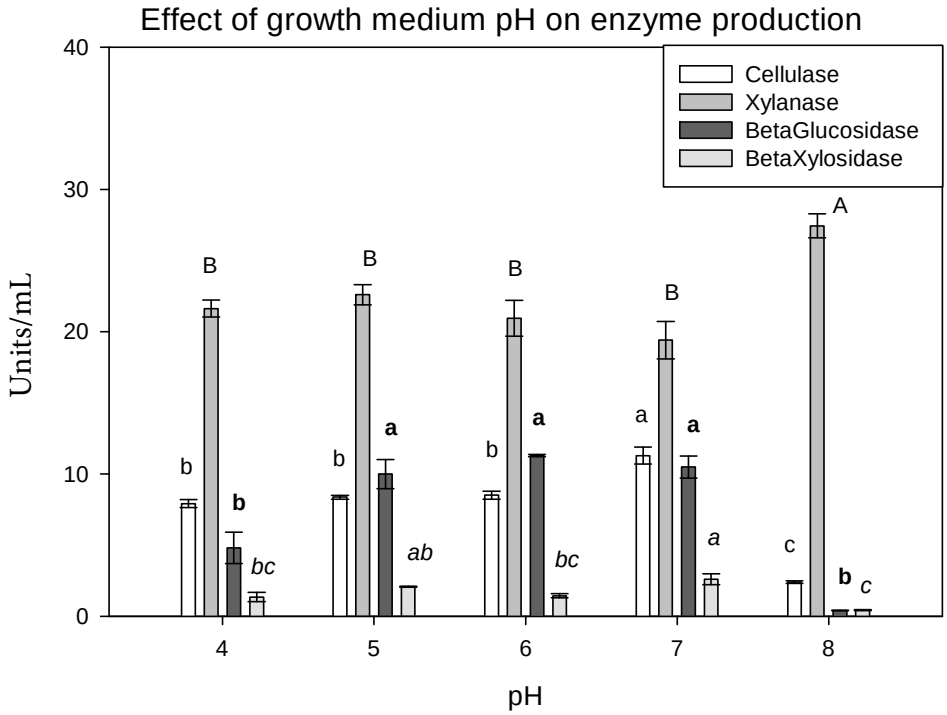

Figure 5: Effect of growth medium pH on enzyme activity. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

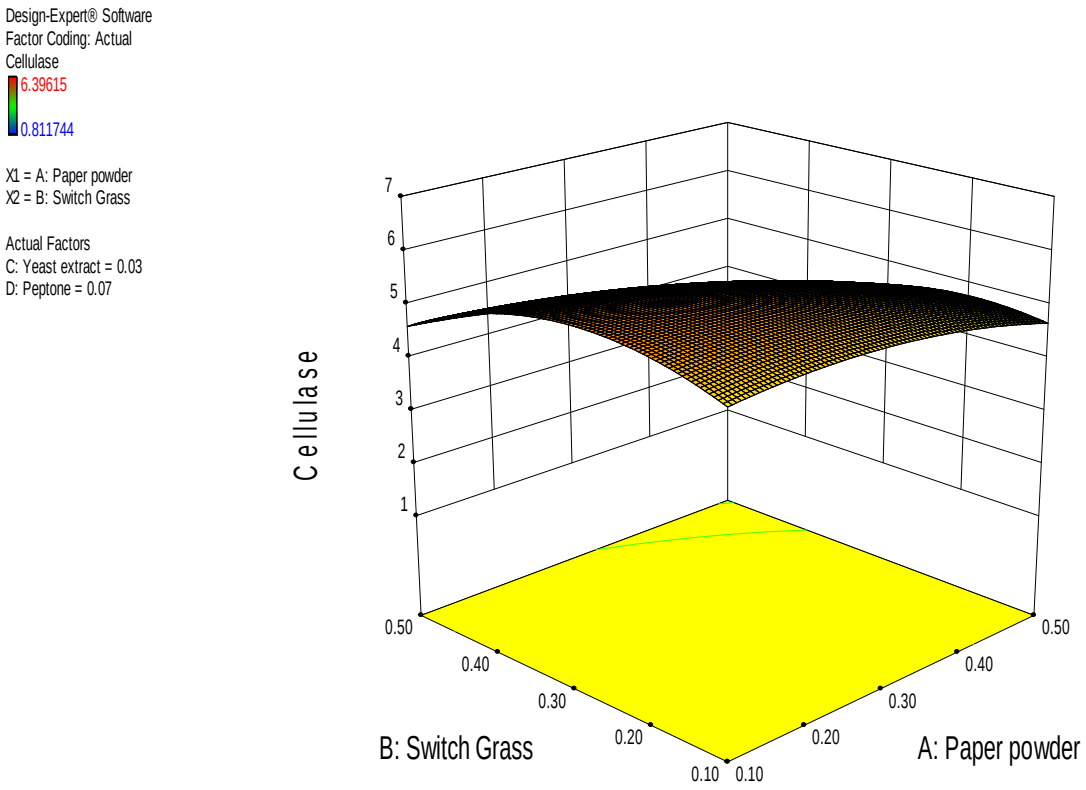

Figure 6: Response surface and contour plot for cellulase activity

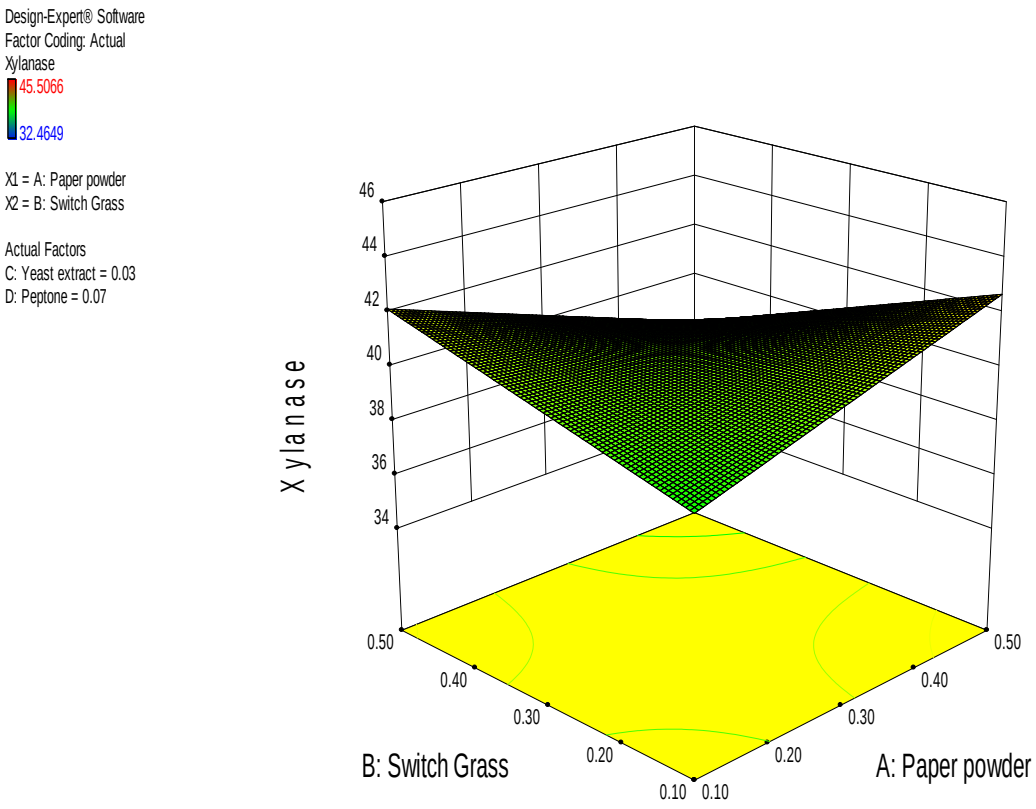

Figure 7: Response surface and contour graphs for xylanase

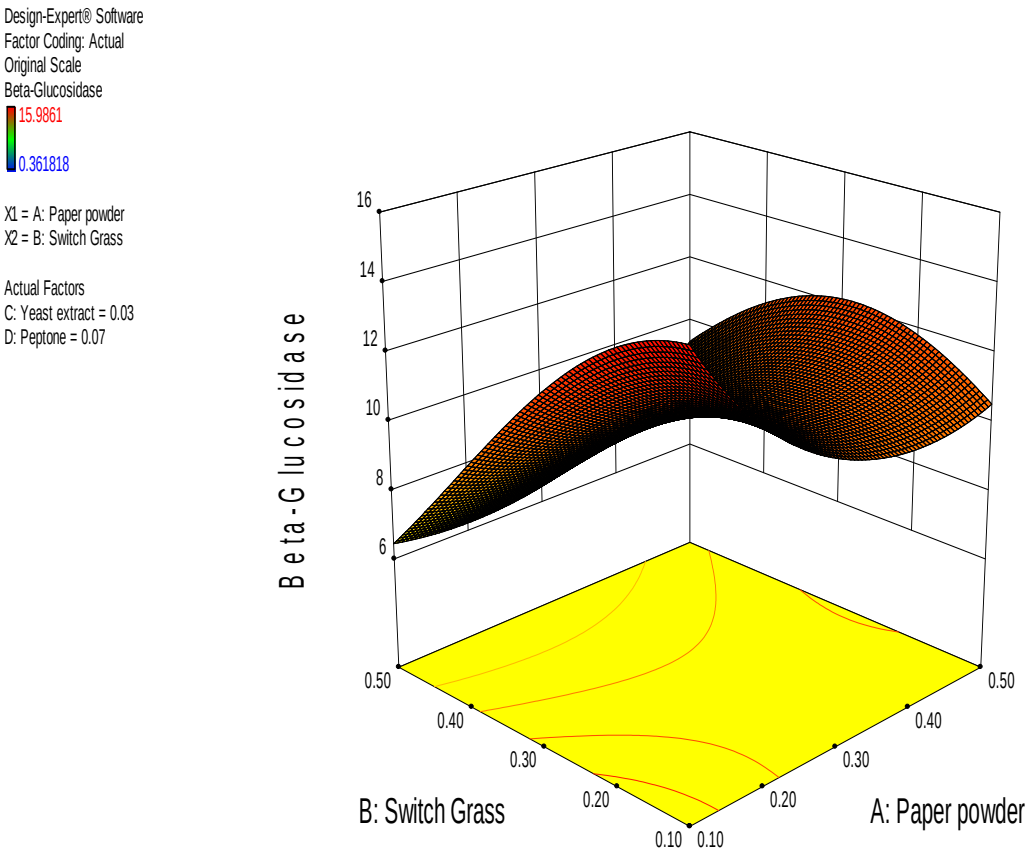

Figure 8: Response surface and contour graphs for β-glucosidase

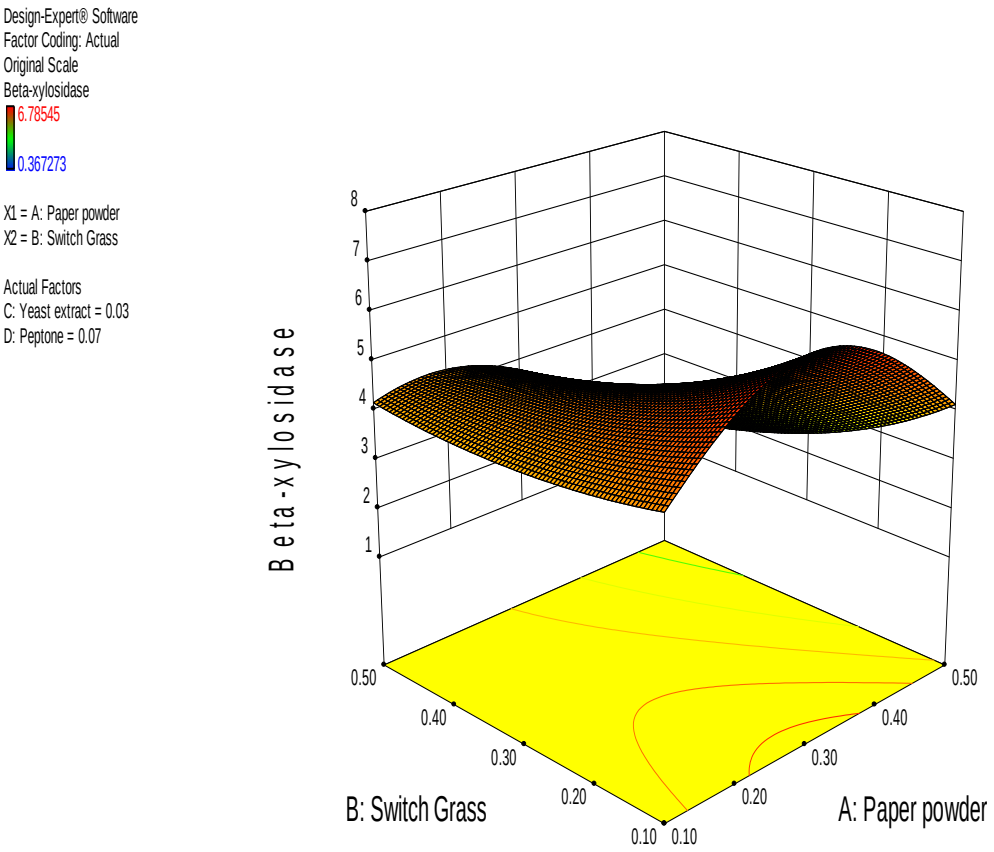

Figure 9: Response surface and contour graphs for β-xylosidase

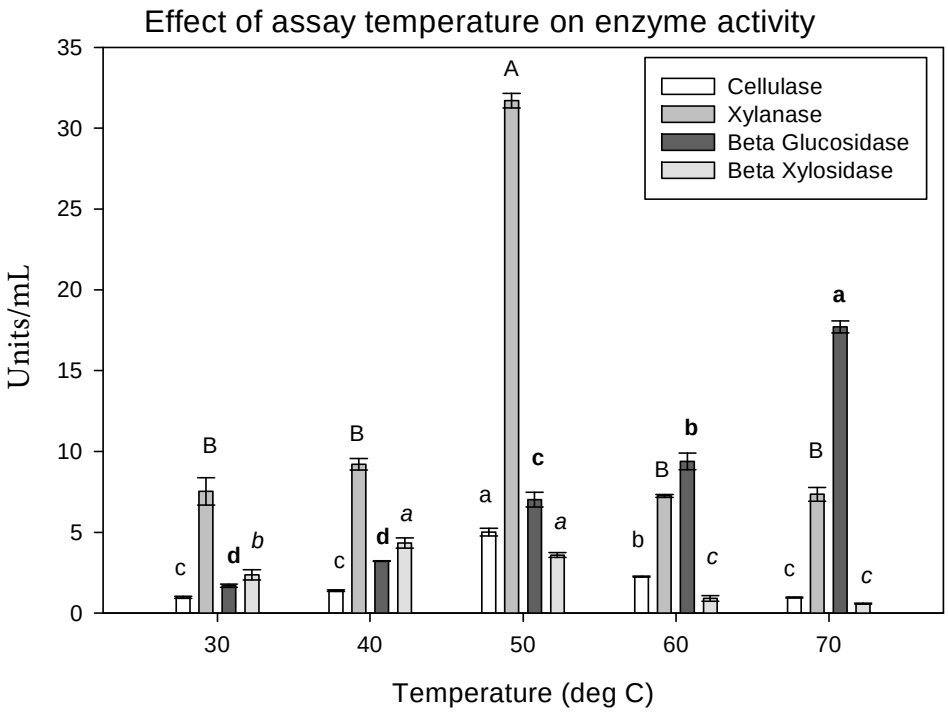

Figure 10: Effect of assay temperature on enzyme activity. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

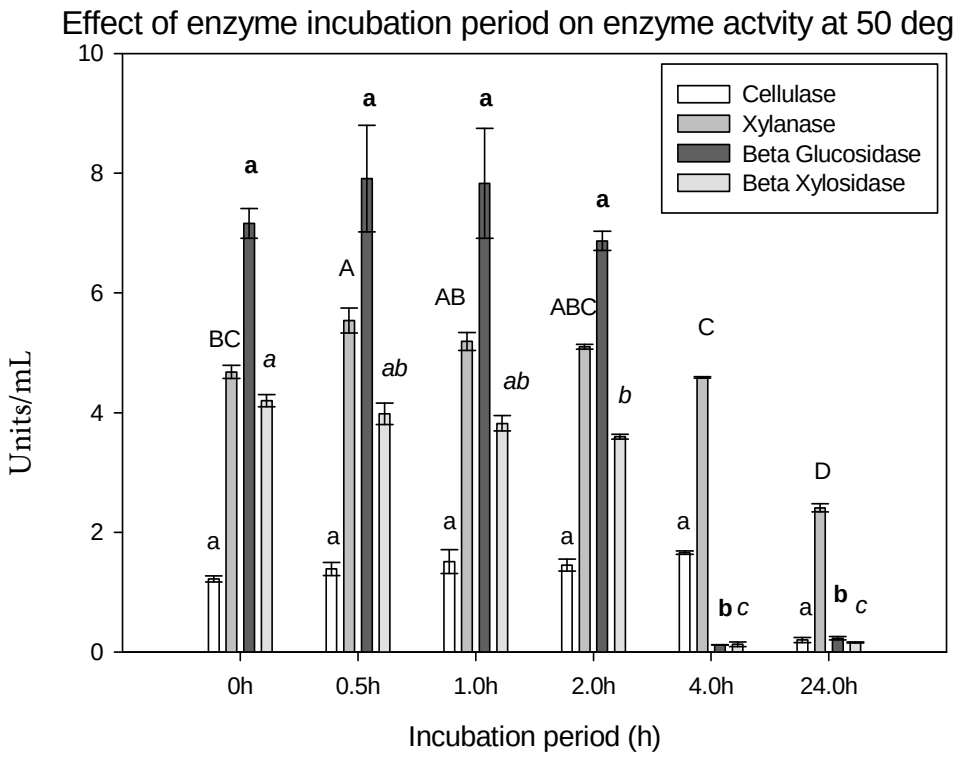

Figure 11: Effect of assay incubation time on enzyme activity at 50 deg C. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

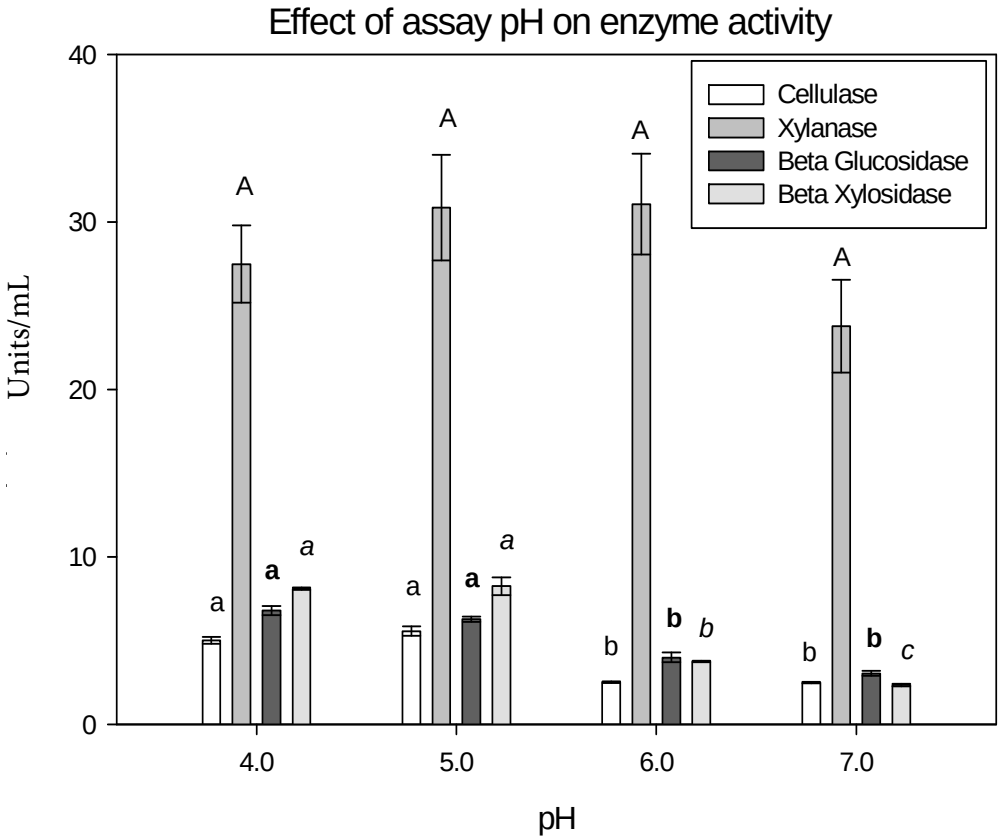

Figure 12: Effect of assay pH on enzyme activity. Pair-wise differences (Tukey) are indicated by lower-case letters for cellulase, upper-case for xylanase, bold for β-glucosidase and italisiced for β-xylosidase. Significance was set at P=0.05

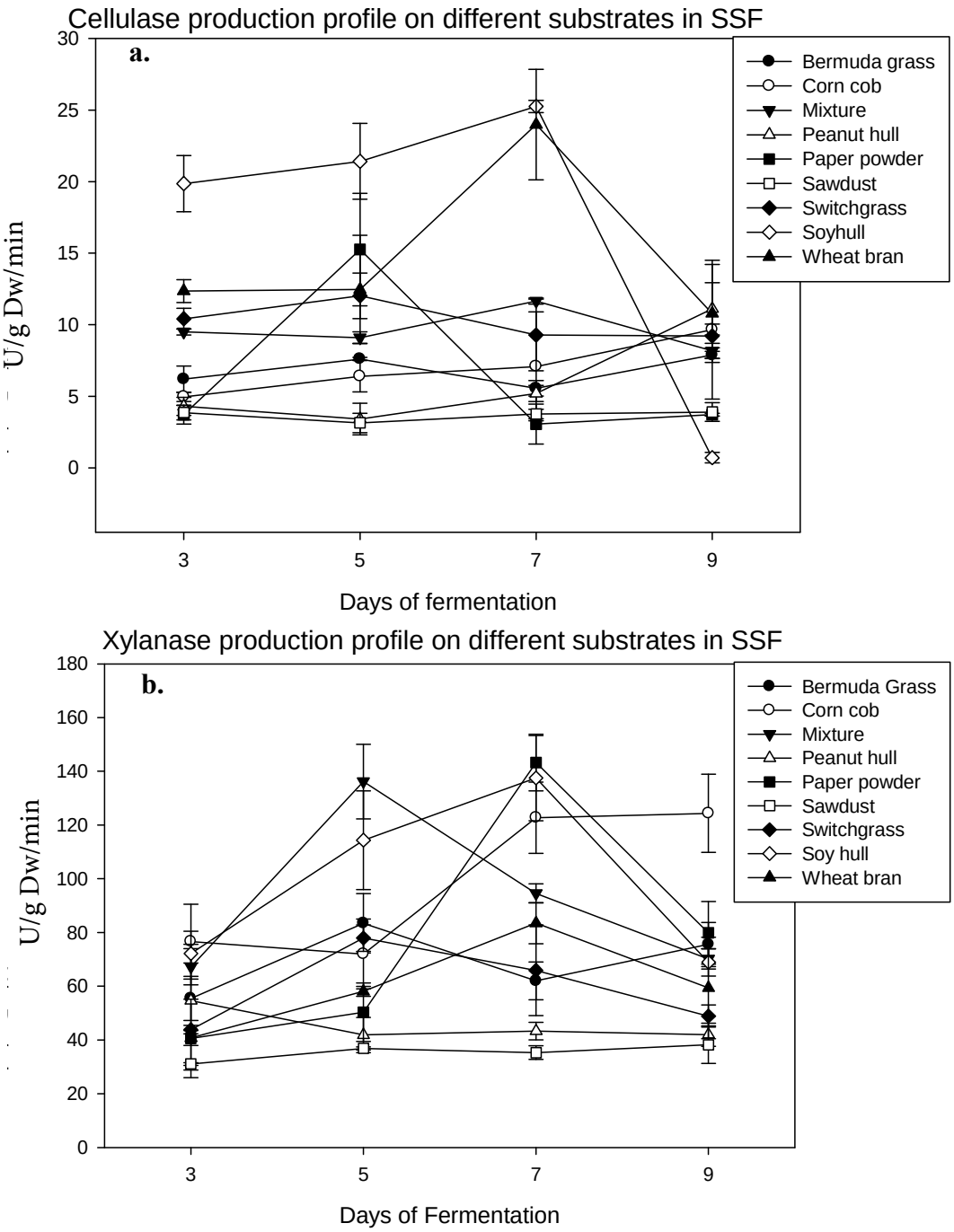

Figure 13: Enzyme production on SSF. a. Cellulase b. Xylanase

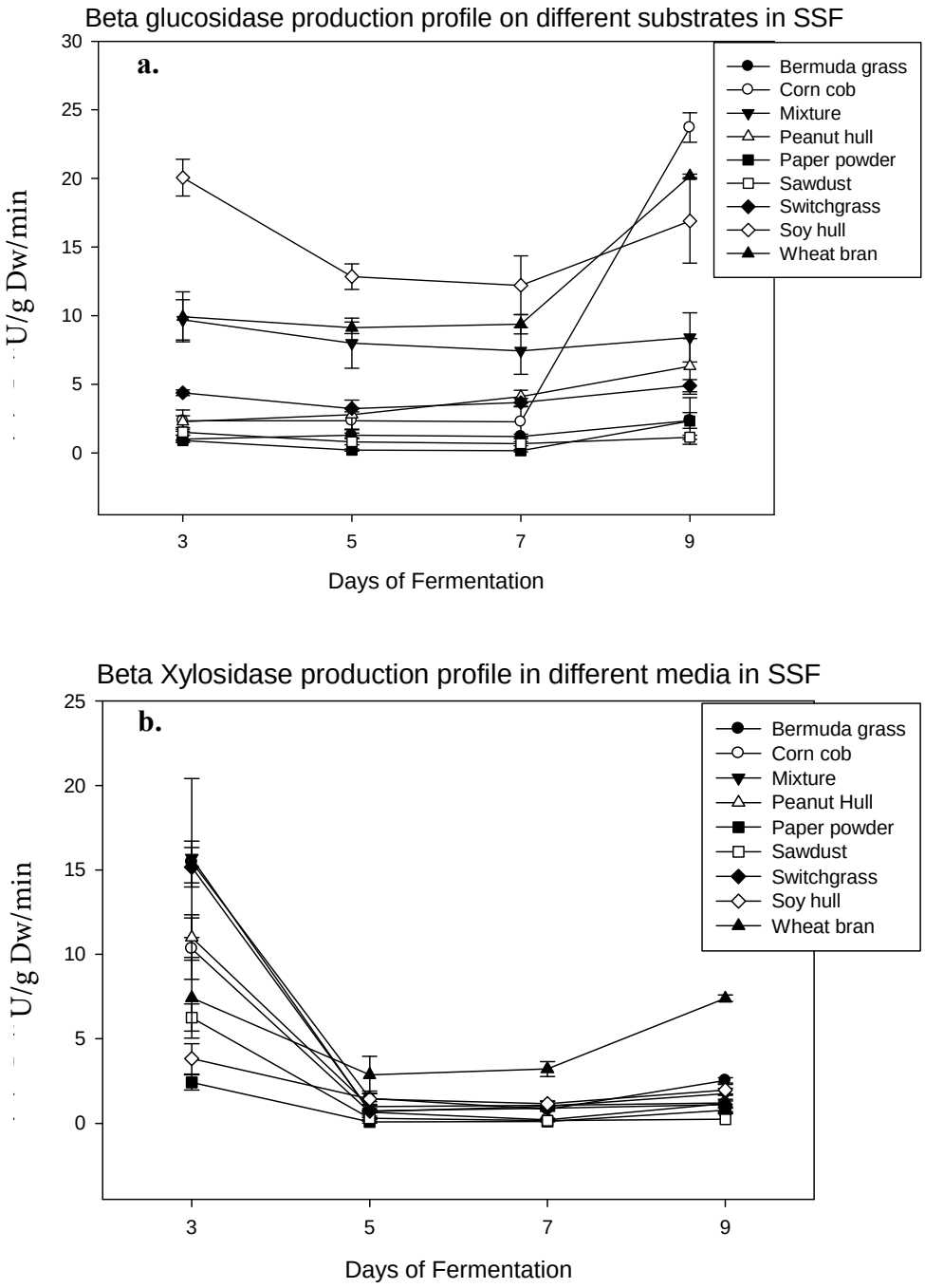

Figure 14: Enzyme production on SSF. a. beta glucosidase b. beta xylosidase

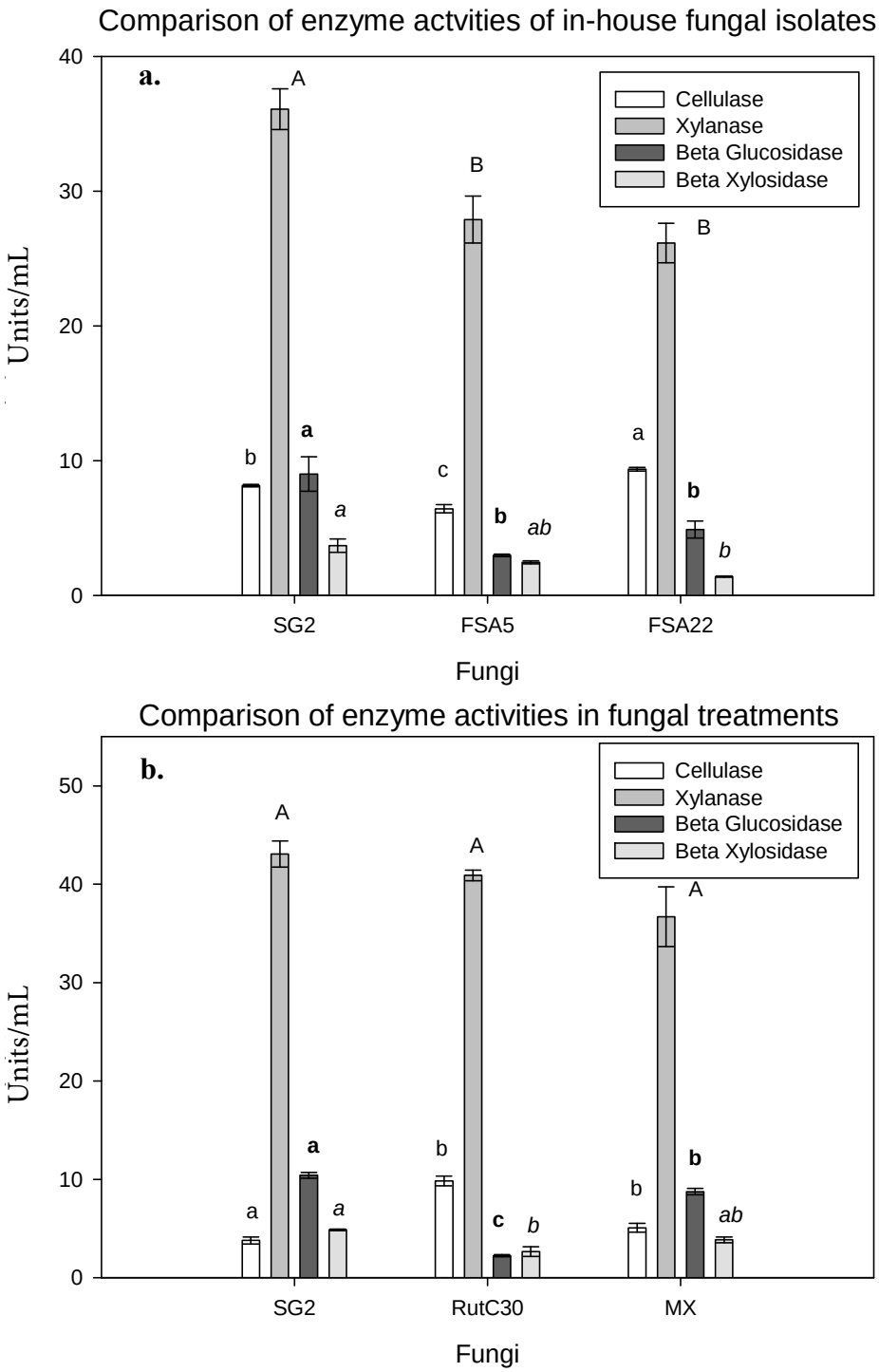

Figure 16: Comparison of enzyme production by different fungi. a. In-house isolates b. SG2 compared with RutC30 and their mixture.

Discussion

Based on the optimization of enzyme production from *Trichoderma* SG2 in SmF, it was found that the control (screening) medium containing 0.5% each of substrates switchgrass and paper powder at 1:1 was ideal for enzyme production. Interestingly, the control medium contained the optimal nitrogen in the form of 0.1% peptone, 0.05% yeast extract and 0.12% ammonium sulfate. The mixture of organic and inorganic nitrogen source seems to be ideal for enzyme activity compared to sole organic or inorganic source. Tween-20 seems to be a better choice than Tween-80 used in the control medium, although the difference is insignificant. Temperature of 30^{o}C and 7.0 pH are optimal for enzyme production. In conclusion, control (screening) medium containing a mixture of paper powder and switchgrass along with nitrogen in the form of peptone, yeast extract and ammonium sulfate under *Tricoderma* sp. SG-2 submerged fermentation for 4 days at 30ºC and pH 7.0 results in optimal production of cellulase, xylanase, β-glucosidase and β-xylosidase, enzymes that are required for saccharification of lignocellulosic biomass for ethanol production. The concentrations of major ingredients in screening medium were further optimized using RSM which suggested that the concentrations of all four ingredients need to be increased: paper powder from 0.5% to 0.62%, switchgrass 0.5% to 0.96%, yeast extract 0.05 to 0.06% and peptone 0.1% to 0.14% to obtain predicted enzyme activities of 4.29 U/ml cellulase, 40.25 U/ml of xylanase, 7.37 U/ml of β-glucosidase and 3.88 U/ml of β-xylosidase under the assay condition. Although RSM suggested other models with similar desirability of 1.0 using higher concentrations of paper powder and lower concentration of switchgrass, we believe that the converse is more useful from a practical standpoint: switchgrass is more likely to be readily available at the farm level than paper powder. However, the predicted responses at optimal concentrations need to be validated and will be conducted in the next quarter.

Enzyme assay conditions like temperature and pH and stability of enzymes were evaluated and it was found that the optimal assay temperature is 50ºC. Only β-glucosidase showed significant activity at 70ºC but could not sustain beyond 30 min (data not shown). Assay pH of 5.0 is optimal for all enzymes. The activities of all enzymes were seen even after 24h. This indicates that the enzymes can be used for saccharification of lignocellulosic biomass for at least 24h at 50ºC. However, saccharification needs to be evaluated separately and the length of saccharification depends on other factors like the target sugar content of 25g in 200g of substrate per liter. This will be evaluated in the next quarter.

Mycelial fungi grow well on SSF and is ideal for producing secondary metabolites (Singhania et al 2009), in additional studies we evaluated eight substrates and their mixture for enzyme production. Irrespective of the substrate, the highest enzyme activities in SSF were compared with respective activities on SmF. Interestingly, *Trichoderma* SG2 on SSF yielded 150 times more cellulase, 180 times xylanase, 30 times β-glucosidase and 60 times the β-xylosidase than that in SmF. The best media and day of fermentation for each enzyme was different. However, based on statistical analyses of our data and practical considerations, SSF of soy hull for three days is ideal for enzyme production. Further validation can help identify the best substrate and its concentration for maximum enzyme activity.

The enzyme activities of in-house isolates SG2, FSA5 and FSA22 were compared. *Trichoderma* SG2 was found to be the highest producer of at least three of the four enzymes. Similarly, the enzyme activities of commonly used *Trichoderma reesi* RutC30, *Trichoderma* SG2 and their mixed culture were compared. Comparatively, SG2 produced significantly higher xylanase, β-glucosidase and β-xylosidase than RutC30, whereas RutC30 produced significantly higher cellulase than SG2. The mixed culture of SG2 and RutC30 was hypothesized to yield the highest activities for all four enzymes. However, this was not the case and rules out the use of mixed culture of SG2 and RutC30 for enzyme production. The growth rate of SG2 and RutC30 were not comparable, with fast growing SG2 overtaking RutC30 the likely reason for the average performance of mixed culture.

Milestone (A.1.ML.1): Complete selection of fungi.

Our results thus far are promising and *Trichoderma* SG2 isolate seems better than the commonly used RutC30 for enzyme production. The comprehensive data collected so far for SG2 suggests that the organism and the process of enzyme production in SSF are intellectually meritorious and are patentable. We firmly believe that the saccharification data that we will collect in the following quarter will further strengthen the case. We anticipate drafting a provisional patent application in the following quarter. Target for milestone for the selected fungus is approximately 25g sugar/200g cellulose-hemicellulose in biomass/L of buffer. Results will be reported in the next quarter.

Task B: Select C5-sugar fermenting yeasts

Introduction

Microbial production of ethanol from lignocellulosic biomass involves the enzymatic breakdown of lignocellulosic biomass to sugars followed by the conversion of sugars to ethanol. Microbial degradation of lignocellulosic biomass primarily yields glucose and xylose. Glucose and xylose are the most abundant sugars in lignocelluloses. Commonly isolated yeast strains like *Saccharomyces* species can efficiently convert glucose to ethanol, whereas only a few yeasts such as *Pichia* sp. can convert xylose to ethanol. In an effort to isolate and characterize yeasts that can ferment xylose to ethanol, soil-decaying biomass composite samples and fruits were collected from several sites in Alabama, and xylose utilizing microbes were isolated by enrichment in high xylose medium.

Methods:

Enrichment of xylose fermenting yeast from soil-decaying biomass composite sample

Soil-decaying biomass (SDB) composite samples were those employed for selection of lignocellulose degrading fungi (second quarter report). The enrichment medium consisted of yeast extract, 10g; peptone, 20g; xylose, 10g; chloroamphenicol, 0.075g per liter of distilled water (YPX). Five milliliters of the medium was dispensed in 16 x 125 mm tubes, capped and autoclaved at 110°C for 10 minutes. After cooling to room temperature, enrichment broths were inoculated with 0.25g of composite biomass-soil sample. Cultures were incubated statically at 30°C for 3 days (round 1).

Thereafter, 0.25 mL was transferred to fresh enrichment medium and further incubated statically at 30°C for 3 days (round 2). Cultures were serially diluted (to 10^{-1} to 10^{-5}) in sterile normal saline (0.9 % w/v) in 16 x 125 mm tubes and 0.1 mL of each dilutions 10^{-2} to 10^{-5} was plated on the enrichment medium solidified with agar (YPX agar). Inoculated plates were incubated at 30°C for 3-4 days. Cultures were examined for yeast-like xylose-utilizing colonies. Selected discrete colonies were further purified by repeated streaking on YPX agar.

Enrichment of xylose fermenting yeasts from fruits.

A wide-scale sampling of locally-grown fruits was collected in Central Alabama. During the time of scheduled sample collection, the region was experiencing a severe to extreme drought, which slowed the rate of produce being brought to market. Many fresh fruits sold in farmer's markets were thus imported from Florida and Georgia, and as far away as California. However, by visiting farmers markets and questioning local produce growers and visiting local growers, we were able to sample only locally-grown fruits. Note the inclusion of items such as peppers okra, and squash in the sampling protocol. While in common parlance, these are called "vegetables", they are technically fruits in a botanical sense, as they are the seed-bearing structures which mature from pollinated flowers. Fifty-four fruit samples were collected (Table 16a&b). Fruit slurry was prepared as follows. Briefly, 5-10 pieces/chunks of each fruit were removed from 3-5 fruits using sterile metal spatulas. The fruit pieces were mashed using pestle and mortar or crushed in sterile beakers. Sterile distilled water was added to chopped fruits in a ratio of 1:1 (1 mL water for every gram of fruit) except that for okra and purple hull peas, 2 mL water for every gram of fruit was added. The enrichment medium consisted of the following (in g/L): yeast extract, 10g; peptone, 20g; xylose, 10g; chloramphenicol, 0.075g and 5 ml of medium was dispensed in 16 x 125 mL tubes, capped and autoclaved at 110°C for 10 minutes. After allowing media to cool to room temperature, replicate tubes were inoculated with 1 mL of fruit slurry. Initial culture density was read at 600 nm before incubation at 30°C. Cultures were examined for yeast-like xylose-utilizing colonies. Selected discrete colonies were further purified by repeated streaking on YPX agar.

Table 16a: Fruit samples for isolation of xylose utilizing yeasts

Sample	date	location		City	Lat	Long	fruit sampled
ALF060911-1	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		blackberries
ALF060911-2	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		red plum
ALF060911-3	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		semi-sweet plum
ALF060911-4	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		tart plum
ALF060911-5	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		banana pepper, sweet
ALF060911-6	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		banana pepper, hot
ALF060911-7	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		blueberries
ALF060911-8	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		peaches
ALF060911-9	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		tomato
ALF060911-10	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		tomato
ALF060911-11	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		yellow squash
ALF060911-12	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		cucumber
ALF060911-13	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		cucumber
ALF060911-14	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		corn
ALF060911-15	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		field peas
ALF060911-16	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		lima beans
ALF060911-17	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		purple hull peas
ALF060911-18	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		eggplant
ALF060911-19	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		okra
ALF060911-20	6/9/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		green beans
ALF061611-1	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		purple hull peas
ALF061611-2	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		rattlesnake snap pea
ALF061611-3	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		cherry tomato
ALF061611-4	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		cowhorn pepper
ALF061611-5	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		cantaloupe
ALF061611-6	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		bell pepper
ALF061611-7	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		green tomato
ALF061611-8	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		white nectarine
ALF061611-9	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		nectarine
ALF061611-10	6/16/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033		zucchini squash

Table 16b: Fruit samples for isolation of xylose utilizing yeasts

ALF062311-1	6/23/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033	tomato
ALF062311-2	6/23/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033	tomato
ALF062311-3	6/23/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033	jalapeno pepper
ALF062311-4	6/23/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033	bell pepper
ALF062311-5	6/23/2011	Curb Market	1004 Madison Ave.	Montgomery, AL	32.379708,-86.295033	bell pepper
ALF071411-1	7/14/2011	Autaga Hill Farms	152 Co Rd. 27	Prattville,AL	32.423017,-86.539049	Asian pears
ALF071411-2	7/14/2011	Autaga Hill Farms	152 Co Rd. 27	Prattville,AL	32.423017,-86.539049	peaches
ALF071411-3	7/14/2011	Blueberry Hill Farm	704 B;ueberry Hill Rd.	Prattville, AL	32.506287,-86.596942	blueberries (on bush)
ALF071411-4	7/14/2011	Blueberry Hill Farm	704 B;ueberry Hill Rd.	Prattville, AL	32.506287,-86.596942	blueberries on ground
ALF071411-5	7/14/2011	Horn of Plenty Fruit farm	Hwy 82	Maplesville, AL	32.655879,-86.768281	tomato
ALF071411-6	7/14/2011	Horn of Plenty Fruit farm	Hwy 82	Maplesville, AL	32.655879,-86.768281	peaches
ALF071411-7	7/14/2011	Horn of Plenty Fruit farm	Hwy 82	Maplesville, AL	32.655879,-86.768281	plums
ALF071411-8	7/14/2011	Horn of Plenty Fruit farm	Hwy 82	Maplesville, AL	32.655879,-86.768281	nectarine
ALF071411-9	7/14/2011	Horn of Plenty Fruit farm	Hwy 82	Maplesville, AL	32.655879,-86.768281	yellow squash
ALF071411-10	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	okra
ALF071411-11	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	yellow squash
ALF071411-12	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	cucumber
ALF071411-13	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	peppers
ALF071411-14	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	peaches
ALF071411-15	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	plums
ALF071411-16	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	peaches
ALF071411-17	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	plums
ALF071411-18	7/14/2011	Harrison Fruit farms	299 Co. Rd. 15	Maplesville, AL	32.69501,-86.796273	peppers

Screening of yeasts for xylose conversion to ethanol in liquid medium

Yeast colonies from enrichment plates were used to inoculate culture tubes filled with 10-12 mL of fermentation medium (g/L distilled water): xylose, 100g; peptone, 3.5g; yeast extract, 3g; KH_2PO_4, 2g; $(NH_4)_2SO_4$, 1g and $MgSO_4.7H_2O$, 1g. Tubes were capped and autoclaved at 110°C for 10 minutes. After the tubes cooled to room temperature, 3-5 loops of selected yeasts were used to pitch the culture and incubated at 25°C for 7 days. Cultures were then qualitatively assessed for ethanol levels using R-Biopharm ethanol analysis kit (catalog number 10 176 290 035), according to the manufacturer's instructions with slight modification. Reagent solution was prepared as follows: each tablet from kit (bottle 2) was dissolved in 3 ml of solution 1(bottle 1) and 0.05 ml of suspension 3 (bottle 3) was added and mixed. Reagent solution (1 ml) was transferred to 1.5 mL semi micro cuvette for each sample in triplicate. Initial absorbance (A_1) was read at 340 nm. Thereafter, the reaction was started by adding 34 µl of ten times diluted sample to each sample reagent solution in cuvette. Final absorbance (A_2) for samples and blanks were read at 340nm after standing for 5-10 minutes at room temperature. Three blanks were prepared in the same way to

determine background effect except that 34 ul of distilled water was added in place of the sample. Ethanol content (g/l) of sample and blank were calculated. Values similar to the blank were not significant.

Results and Discussion

Over 100 yeast isolates from SDB and fruit samples were examined for xylose conversion to ethanol in culture. Xylose fermenting yeasts isolated from fruits displayed higher ethanol content compared to those from SDB. Based on ethanol content of cultures, twenty yeast isolates were shortlisted for optimization of xylose conversion to ethanol using lignocellulose hydrolysate. The isolates will be further evaluated for resistance to inhibitory products of lignocellulose hydrolysis as well as ethanol tolerance. Ethanol levels in xylose fermentation cultures were low. Nonetheless, the screening procedure was qualitative. Twenty shortlisted isolates are being optimized for xylose conversion to ethanol. Pigmented yeast isolates are currently being assessed for their lipid and fatty acid content for potential application in biodiesel production.

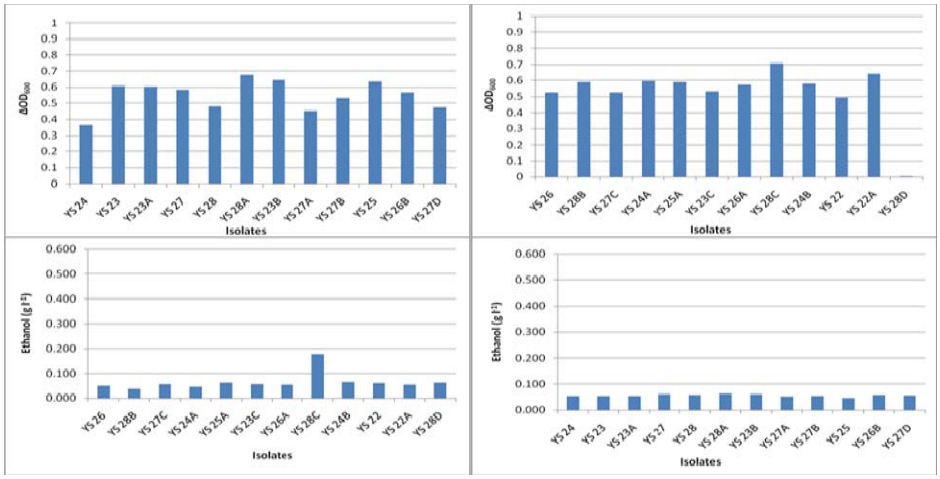

Figure 1: Growth (ΔOD) and ethanol (g l^{-1}) in xylose culture of isolates from SDB. Ethanol, < 0.1 g l^{-1} was not significant.

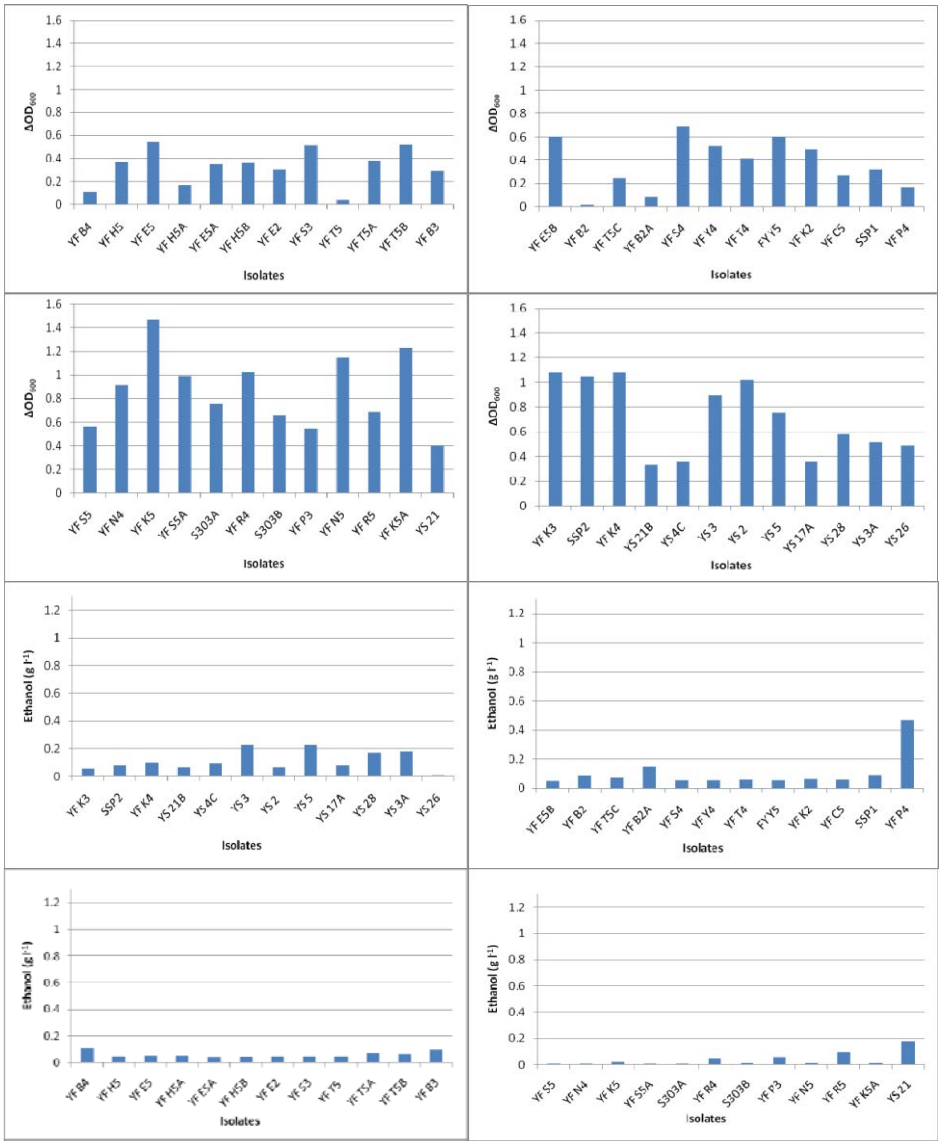

Figure 2: Growth (ΔOD) and ethanol (g l^{-1}) in xylose culture of isolates from SDB. Ethanol, < 0.1 g l^{1} was not significant.

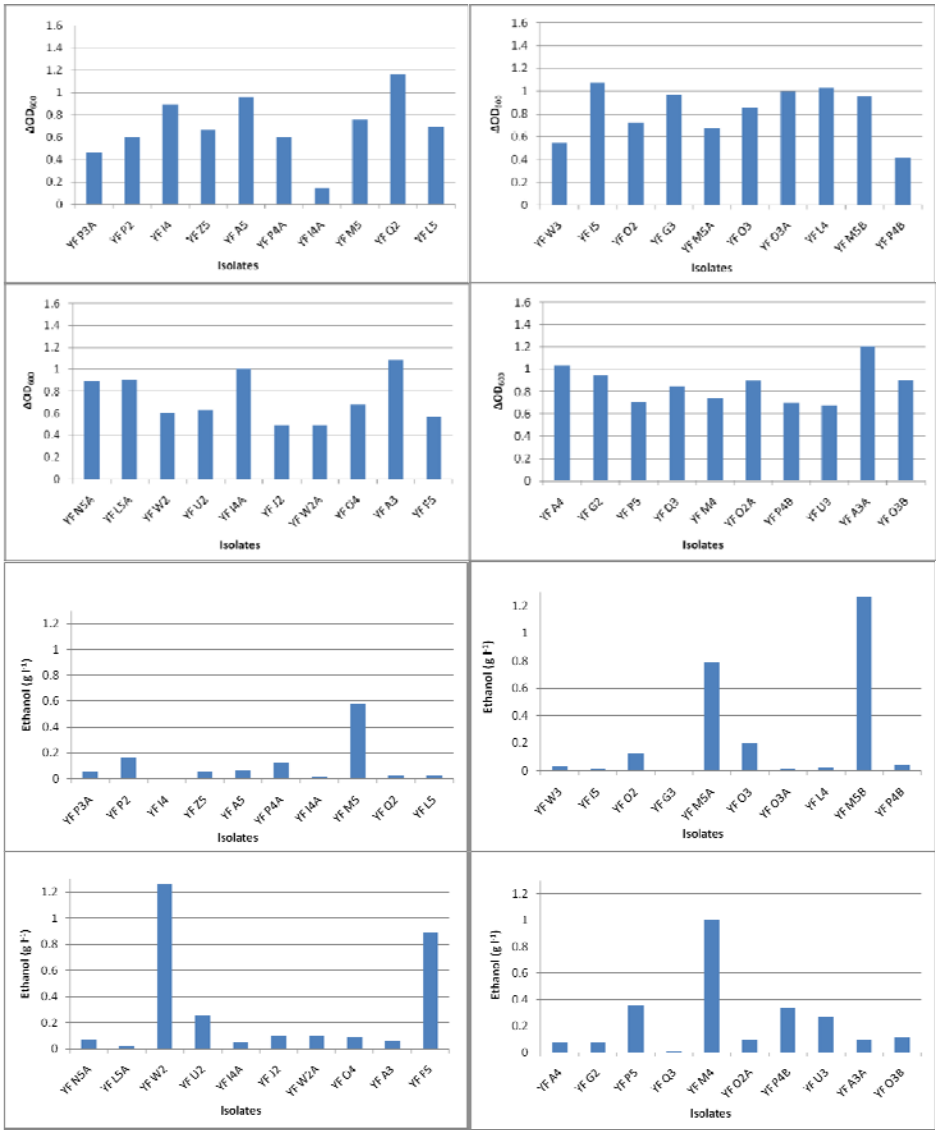

Figure 3: Growth (ΔOD) and ethanol (g l^{-1}) in xylose culture of isolates from fruits. Ethanol, < 0.1 g l^{-1} was not significant.

Milestone (A.1.ML.1): Complete selection of fungi.

We isolated xylose fermenting yeasts. Xylose fermentation by selected yeasts is under optimization. We will combine xylose and glucose fermenting yeasts in fermentation studies. Selected yeasts are expected to produce 45 g ethanol from 100 g of xylose or glucose per liter. More studies are ongoing in order to assess this milestone. Results will be reported in the next quarter.

Presentations:

1. Nanjundaswamy, A. and Okeke, B.C. (2011). Optimization of culture parameters for production of cellulolytic and xylanolytic enzymes from a new *Trichoderma* species SG2. Society for Industrial Microbiology, Annual Meeting (New Orleans, Louisiana, July 24-28).
2. Okeke, B.C., Deravi, Y., Prescott, A., Bishop, J., Sawyer, L., Peaks, S., Nanjundaswamy, A. and Hall, R (2011). Screening of xylose-utilizing yeasts from soil-decaying biomass composite samples and fruits for ethanol production. Society for Industrial Microbiology, Annual Meeting (New Orleans, Louisiana, July 24-28).
3. Okeke, B.C. (2011): Farm Deployable Microbial Bioreactor for Fuel Ethanol Production. Auburn University Board of Trustees (02/14/2011).
4. Andrew Prescott, Yasi Deravi, Shanticia Peaks, Jamie Bishop, Leah Sawyer, Rosine Hall and Benedict Okeke (2011). Scereening of xylose-utilizing yeast isolates for ethanol production from biomass (Poster). Auburn Montgomery Undergraduate Research Symposium.
5. Yasi Deravi, Andrew Prescott, Shanticia Peaks, Jamie Bishop, Leah Sawyer, Rosine Hall and Benedict Okeke (2011). Isolation of plant fiber-degrading fungi for conversion of lignocelluloses to sugars. Auburn Montgomery Undergraduate Research Symposium.

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10. Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. Biotechnology Progress 21:816-822.
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12. Singhania RR, Patel AK, Soccol CR, Pandey A (2009) Recent advances in solid-state fermentation *Biochemical Engineering Journal* 44: 13-18.

PART 3: Research Activities of FY-11 (July 1, 2011 to September 30, 2011).
Written by: Benedict Okeke, Ananda Nanjundaswamy and Rosine Hall

Introduction

Previously, crude enzymes from *Trichoderma* SG2 were harvested from solid-state fermentation of wheat bran. The enzymes included cellulase, xylanase, beta-glucosidase and beta-xylosidase each with activities of 23, 36, 8 and 1 units/g dry basis/min respectively. The crude enzymes were produced for saccharifying biomass namely switchgrass. Additionally, previously isolated xylose utilizing yeasts were evaluated for ethanol production.

The following studies were carried out: 1) sodium hydroxide and sulfuric acid pretreatment and saccharification using 5, 10 and 20% crude enzyme; 2) sulfuric acid, ammonia and calcium hydroxide pretreatments followed by saccharification using 50 and 100% crude enzyme; 3) simultaneous saccharification and fermentation (SSF) of biomass namely switchgrass using sulfuric acid pretreatment, 100% crude enzyme with three yeast treatments namely *Saccharomyces cerevisiae*, *Pichia stipitis* NRRL Y-7124 and their mixed culture; 4) ethanol fermentation of at least four isolates of xylose utilizing microbes in comparison with *P. stipitis* and 5) filing of a provisional patent for the farm deployable technology for self-sufficient production of cellulosic ethanol.

Materials and Methods

Pretreatment

Alkali pretreatment

For alkali treatment, NaOH was used. A 2% solution of NaOH was prepared using de-ionized water. Approximately 10g of switchgrass was weighed into a 250ml flask, and 90ml of 2% NaOH was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 121^0C for 60min. After autoclaving, flasks were carefully removed and strained using a muslin cloth to remove liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during alkali hydrolysis. The solid portion (pretreated switchgrass) was washed several times with tap water to remove alkali. The complete removal of the alkali was confirmed by suspending a small portion of the sample in water and pH measured. Pretreated samples were air dried at room temperature for 24 to 48h.

Lime Pretreatment

For lime treatment $Ca(OH)_2$ was used. A 2% solution of $Ca(OH)_2$ was prepared using de-ionized water. Approximately 10g of switchgrass was weighed into a 250ml flask and 90ml of 2% $Ca(OH)_2$ was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 121^0 C for 60min. After autoclaving, flasks were carefully removed and strained using a muslin cloth to remove $Ca(OH)_2$ and liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during alkaline hydrolysis. The solid portion (pretreated switchgrass) was washed several times to remove $Ca(OH)_2$. The complete removal of the $Ca(OH)_2$ was confirmed by suspending a small portion of the sample in water and pH measured. Pretreated samples were air dried at room temperature for 24-48h.

Acid Pretreatment

For acid treatment H_2SO_4 was used. A 2% solution of H_2SO_4 was prepared using de-ionized water. Approximately 10g of switchgrass was weighed into a 250ml flask and 90ml of 2% H_2SO_4 was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 121^0 C for 60min. After autoclaving flasks were carefully removed and strained using a muslin cloth to remove liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during acid hydrolysis. The solid portion (pretreated switchgrass) was washed several times to remove acid. The complete removal of the acid was confirmed by suspending a small portion of the sample in water and pH measured. Pretreated samples were air dried at room temperature for 24-48h.

Ammonia Pretreatment

A 25% solution of ammonium hydroxide (NH_4OH) was prepared using de-ionized water. Approximately 10g of switchgrass was weighed into a 250ml flask and 90ml of 25% NH_4OH was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 120^0 C for 60min. After autoclaving flasks were carefully removed and strained using a muslin cloth to remove liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during ammonia hydrolysis. The solid portion (pretreated switchgrass) was washed several times to remove ammonia. Pretreated samples were air dried at room temperature for 24-48h.

Saccharification

Duplicate samples from different pretreatments were subjected to saccharification using different enzyme loading based on the dry weight of the samples.

Enzyme preparation

Cellulolytic and xylanolytic enzymes from *Trichoderma* SG2 solid state fermentation of soy hull were used for saccharification. Enzymes were extracted using deionized water which contained 2ppm lactrol as an antibacterial agent during saccharification. Extracted enzymes were directly added into the substrate taken in air tight flasks. For saccharification in air tight flasks, about 10g of the pretreated material was mixed with 90ml enzyme extract and caps were closed tightly to prevent loss of moisture. Duplicate flasks were maintained for each enzyme treatment and pretreated material. All flasks were incubated at 50^0C on an orbital shaker maintained at 150rpm. Approximately 100µl of sample was collected from each flask daily up to 96h. Samples were diluted appropriately and centrifuged and filtered using 0.4µm filters before analyzing for sugars using HPLC.

Simultaneous Saccharification and Fermentation (SSF)

Acid pretreated biomass was subjected to SSF using yeasts *Saccharomyces cerevisiae*, *Pichia stipitis* or their mixed culture. As previously optimized, 100% enzyme loading was used for saccharification.

Yeast inoculum preparation

A vial of yeast was inoculated into a 50ml sterile broth in a 250ml flask containing 1% glucose, 0.1% peptone and 0.05% yeast extract. Flasks were incubated at 30^0C for 48h at 200rpm orbital shaker. About 10% of inoculum was used for monoculture fermentation, whereas 5% of each of the yeasts was used for mixed culture fermentation.

SSF
For SSF in air tight flask, about 10g of the pretreated material was mixed with 90ml enzyme extract and 10% yeast inoculum. Flasks were capped tightly to prevent loss of ethanol. Duplicate flasks were maintained for each enzyme treatment and pretreated material. All flasks were incubated at 30^0C on an orbital shaker maintained at 150rpm. Approximately 100µl of sample was collected from each flask daily up to 96h. Samples were subjected for HPLC analysis to determine the release of sugars and production of ethanol.

Control 1 consisted of virgin switchgrass that was untreated and hydrolyzed by 100% crude enzyme extract, whereas control 2 consisted of 2% sulfuric acid pretreated switchgrass with 100% enzyme hydrolysis at 30^0C.

Further Screening of yeasts for xylose conversion to ethanol in liquid medium
Thirty-five more xylose utilizing microbial isolates (Fig. 8) were screened for xylose conversion to ethanol. Culture conditions (D'Amore 1992) and ethanol analysis (Okeke et al. 2011) were as described in the third quarter report.

Selection of xylose utilizing microbial isolates for further studies
Based on repeated screening of microbial isolates for xylose conversion to ethanol, four isolates, RB (RBP5-1), YS (YS28C) JA (JAP4-2) and PC (PCH3-1), were selected for further studies. Microscopic characteristics of isolates RB and JA suggests they are likely xylose fermenting bacteria. Molecular characterization of selected microbial isolates is in progress.

Fermentation medium and conditions
The fermentation medium comprised of the following (g/L): peptone (3.5g); KH_2PO_4 (2.0 g); $NH_4(SO_4)_2$ (1.0 g); $MgSO_4.7H_20$ (1.0 g); yeast extract (3.0 g) and xylose (20g) and was autoclaved at 110°C for 10 minutes. The medium was cooled to room temperature before dispensing 20 mL into sterile 50 mL centrifuge tubes. The inoculum for each organism and *Pichia stipitis* were cells obtained from 7 day old cultures prepared in the fermentation medium that contained 5% xylose. Cells were recovered by centrifuging at 5000 rpm for 10 minutes and re-suspended in 20 mL of fermentation medium. The inoculum suspension (OD_{600} 0.71, 1.36, 0.54, 1.25 and 0.24) was vigorously shaken before transferring 5 mL to triplicate fermentation medium for each organism. The fermentation cultures were statically incubated at 25°C for 5, 10, 15, 20 days before HPLC analysis.

HPLC estimation of sugars and ethanol:
Method outlined in Ananda et al (2011) was used for quantifying sugars and ethanol. Briefly, about 100 µl of the supernatant was diluted 1:10 with water and filtered using 0.45-µm syringe filters and samples were analyzed with a Shimadzu HPLC equipped with refractive index detector and CTO-20A column oven at 82°C. Water was used as the mobile phase with a flow rate of 0.6 ml/min. A Rezex-organic acid column was used to quantify sugars and ethanol.

Statistical analyses
Data were analyzed statistically using SAS (version 9.1.4). Analysis of Variance (ANOVA) was carried out to compare treatments using PROC ANOVA. Pairwise-comparison was carried out using Tukey adjustment and significance was set at P=0.05.

Results

Pretreatment and saccharification of switchgrass

As a preliminary investigation, 5, 10 and 20% crude enzyme was used for saccharification of alkali or acid-pretreated switchgrass (Fig. 1 and 2). The highest glucose release from sodium hydroxide pretreatment was around 0.35g/L at 10 and 20% enzyme loading. Pretreatment incubation time did not affect the sugar release (Fig. 1a). Similarly, highest xylose release was around 0.14g/L at 10 and 20% enzyme load and incubation duration did not significantly affect the sugar yield except at 5% loading (Fig. 1b). Acid pretreatment resulted in 10 times the sugar yield from sodium hydroxide pretreatment. The highest glucose release was 3.6 g/L at 20% enzyme loading and after 72h incubation (Fig. 2a). The sugar yield increased with time and enzyme loading. Similar trend was seen with respect to xylose release (Fig. 2b). The highest xylose release was 2.2g/L at 20% enzyme loading after 72h of pretreatment and saccharification.

To further increase the sugar yield from pretreated biomass, 50% and 100% crude enzyme were used. When 2% sulfuric acid pretreatment was used, the highest glucose yield of 9.7g/L was observed at 72h and was not statistically different from the yield at 96h (Fig. 3a). Similarly, the highest xylose yield was 0.36g/L at 96h with 100% crude enzyme and was not statistically different from that at other time points (Fig. 2b). Crude enzyme at 100% yielded the highest sugar level of 10g/L.

Ammonia pretreatment with 100% enzyme yielded about 1.1g/L of glucose at 48h and was similar to the yield at all other time points (Fig.4a). Similar yield was seen with 50% enzyme at 72h (Fig. 4a). However, the highest xylose yield was 0.2g/L at 24h with 100% enzyme load and was significantly higher than that at 50% enzyme load (Fig. 4b).

Lime pretreatment with 100% enzyme yielded the highest glucose concentration of 4.4g/L at 48h which was not different from yields at 24h or 72h (Fig. 5a). However, 50% enzyme resulted in only half the yield around 2.5g/L at 48h. Xylose yield of 0.1g/L was seen at 72h with 100% crude enzyme (Fig. 5b).

Overall, dilute sulfuric acid pretreatment and 100% crude enzyme resulted in the best saccharification with10g/L of sugar from switchgrass.

Simultaneous saccharification and fermentation

There were two controls in this study: Control 1 contained virgin switchgrass without pretreatment and hydrolyzed with 100% crude enzyme resulted in highest residual glucose of 0.9g/L at 48h (Fig. 6a) and xylose of 0.47 g/L (Fig.6b). Another control, control 2 was switchgrass pretreated with 2% sulfuric acid and hydrolyzed with 100% crude enzyme at 30 ^{0}C resulted in highest residual glucose of 2.7g/L (Fig.6a) and xylose of 2.4g/L (Fig. 6b), both at 24h. However, reduction in sugar content over time was noticed in control 2 which is an indicator of microbial growth, possibly the growth of *Trichoderma* SG2 from the crude enzyme.

As far as residual glucose in yeast treatments are concerned, the highest yield was 1.0g/L at 72h for *S. cerevisiae* and was not different from the highest glucose yields at 48h in *P. stipitis* and 96h with mixed culture (Fig. 6a). However, the highest xylose level of 2.5g/L was seen at

96h in *S. cerevisiae* fermentation and was significantly different from the highest yields of 1.2g/L in *P. stipitis* at 24h and 1.1g/L at 48h in mixed culture fermentation (Fig.6b).

Ethanol production from SSF was the highest at 96h in *P. stipitis* and mixed culture with a yield of 4.4g/L which was different from *S. cerevisiae* yield of 2.4g/L (Fig.6c).

Fermentation of xylose utilizing yeasts

The residual xylose after 5, 10 and 15 days of fermentation are outlined in Fig.7a. *Pichia stipitis* fermentation was used as a control. The residual xylose in *P. stipitis* fermentation was around 12.8g/L on day 10 and was not different from that on day 5 or day 15 (Fig. 7a). Further, the comparison of xylose levels among the five different yeast treatments is outlined in Fig. 7a. The highest ethanol yield from the control namely, *P. stipitis* was 2.6g/L after day 5 and was not different from that on day 10 and day 15 of fermentation (Fig. 7b). On day 5, ethanol yield of control *P. stipitis* was similar to that of the isolate RB. However, on day 10 and 15, ethanol from *P. stipitis* was greater than that from RB and other isolates.

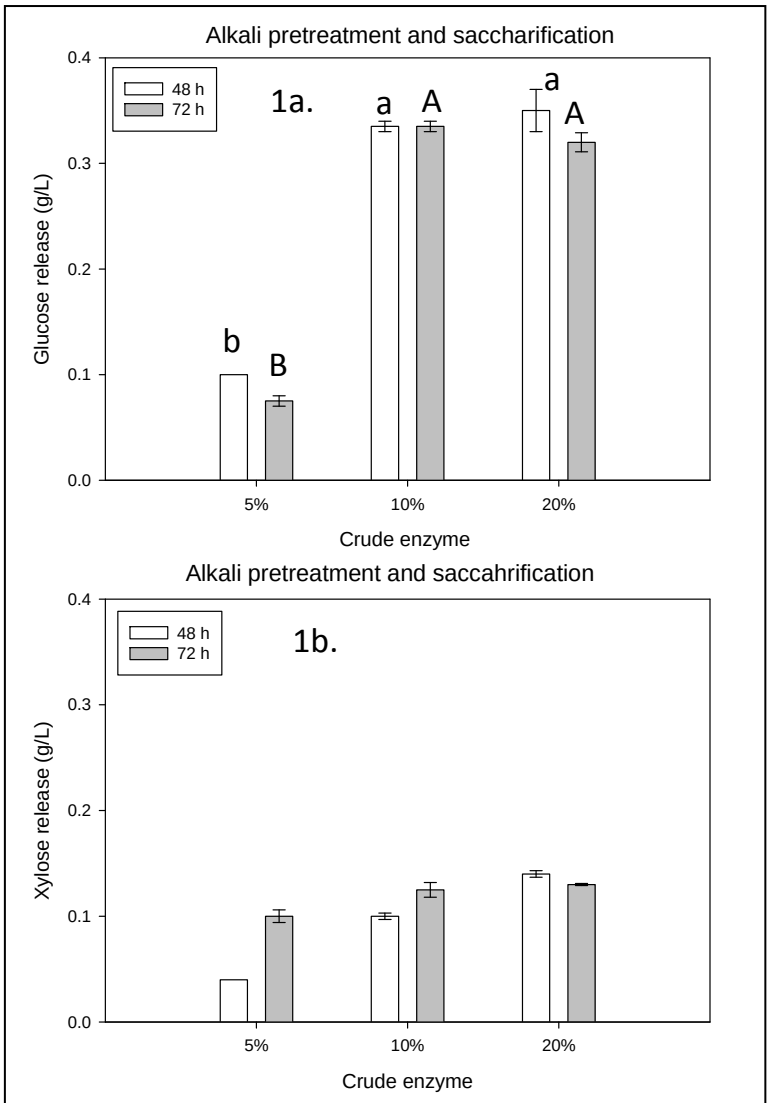


Fig. 1 Sodium hydroxide pretreatment and enzyme hydrolysis. a. Glucose yield b. xylose yield. Lower-case letters compare treatments after 48h incubation and upper-case letters compare treatments after 72h incubation. Treatments that do not share a letter are significantly different from others at P=0.05. Treatments were not different in 1b.

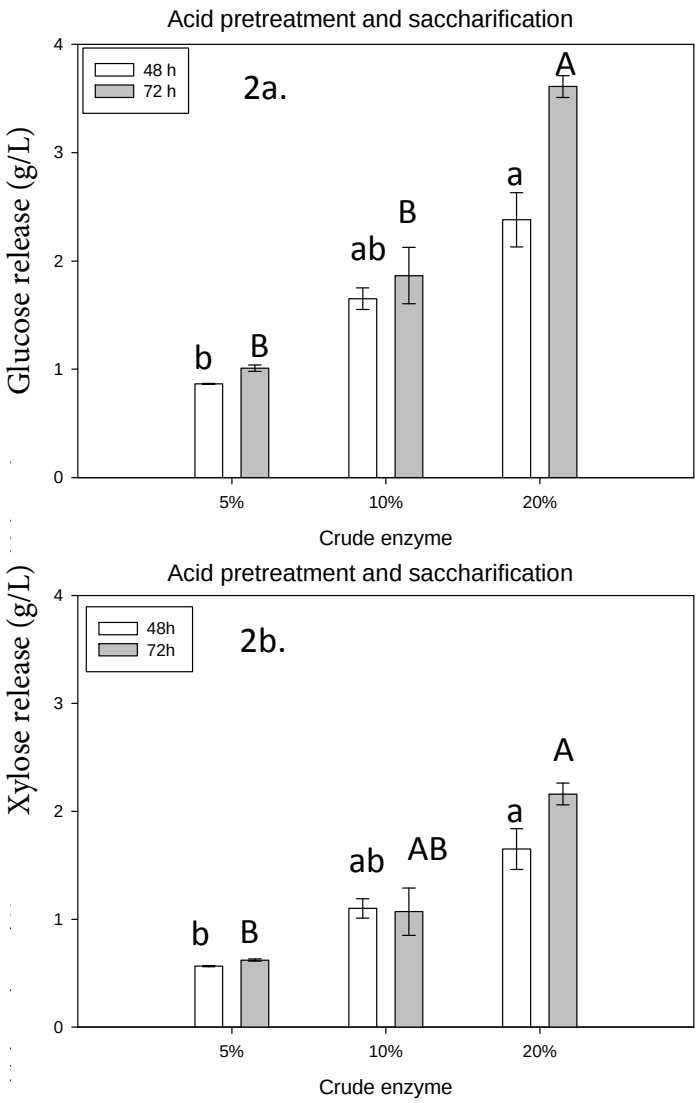


Fig. 2 Dilute sulfuric pretreatment and enzyme hydrolysis. a. Glucose yield b. xylose yield. Lower-case letters compare treatments after 48h incubation and upper-case letters compare treatments after 72h incubation. Treatments that do not share a letter are significantly different from others at P=0.05.

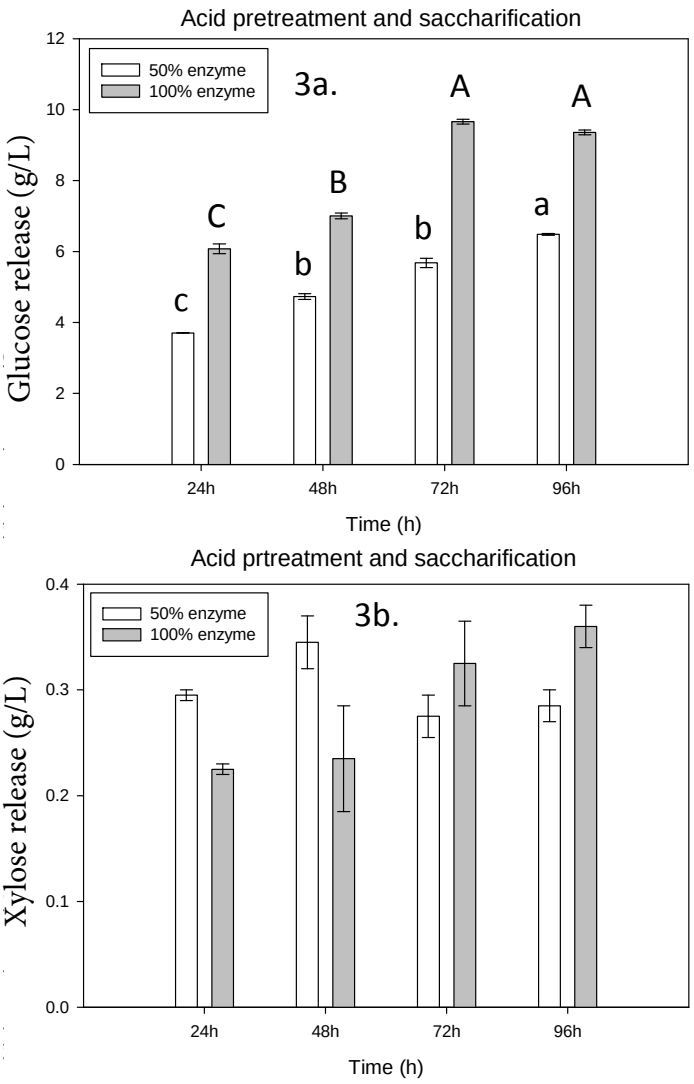
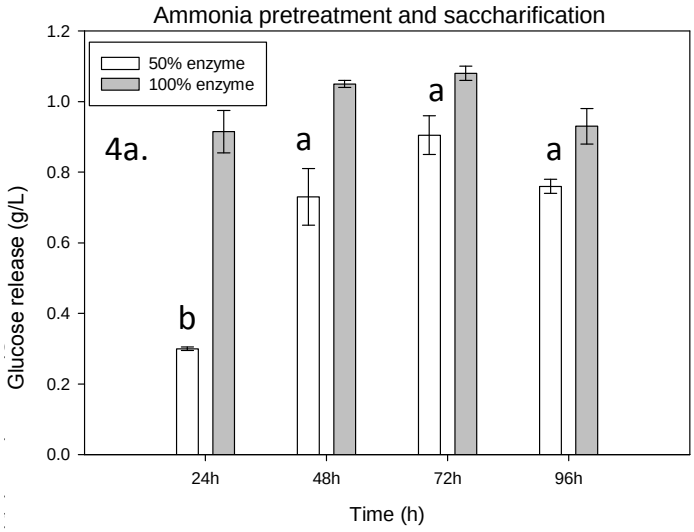


Fig. 3 Dilute sulfuric pretreatment and enzyme hydrolysis. a. Glucose yield b. xylose yield. Lower-case letters compare treatments with 50% crude enzyme hydrolysis and upper-case letters compare treatments with 100% crude enzyme. Treatments that do not share a letter are significantly different from others at P=0.05. Treatments were not significantly different in 3b.



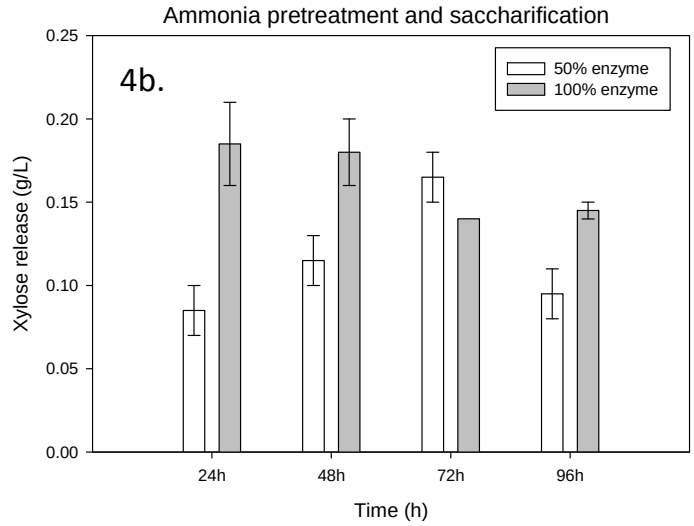
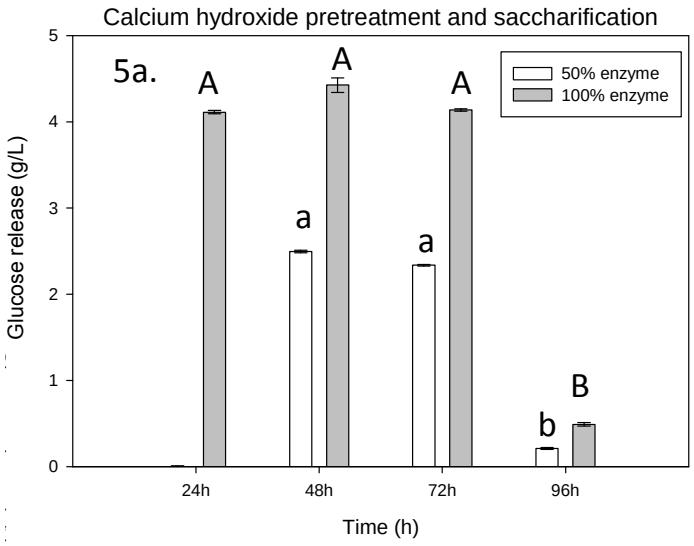


Fig. 4 Ammonia pretreatment and enzyme hydrolysis. a. Glucose yield b. xylose yield. Lower-case letters compare treatments with 50% crude enzyme hydrolysis and upper-case letters compare treatments with 100% crude enzyme. Treatments that do not share a letter are significantly different from others at P=0.05. Treatments were not significantly different in 4b.

Xylose release (g/ L)



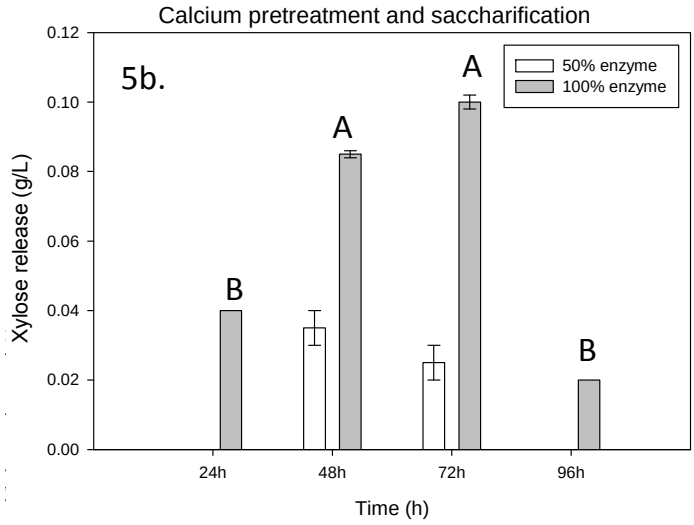
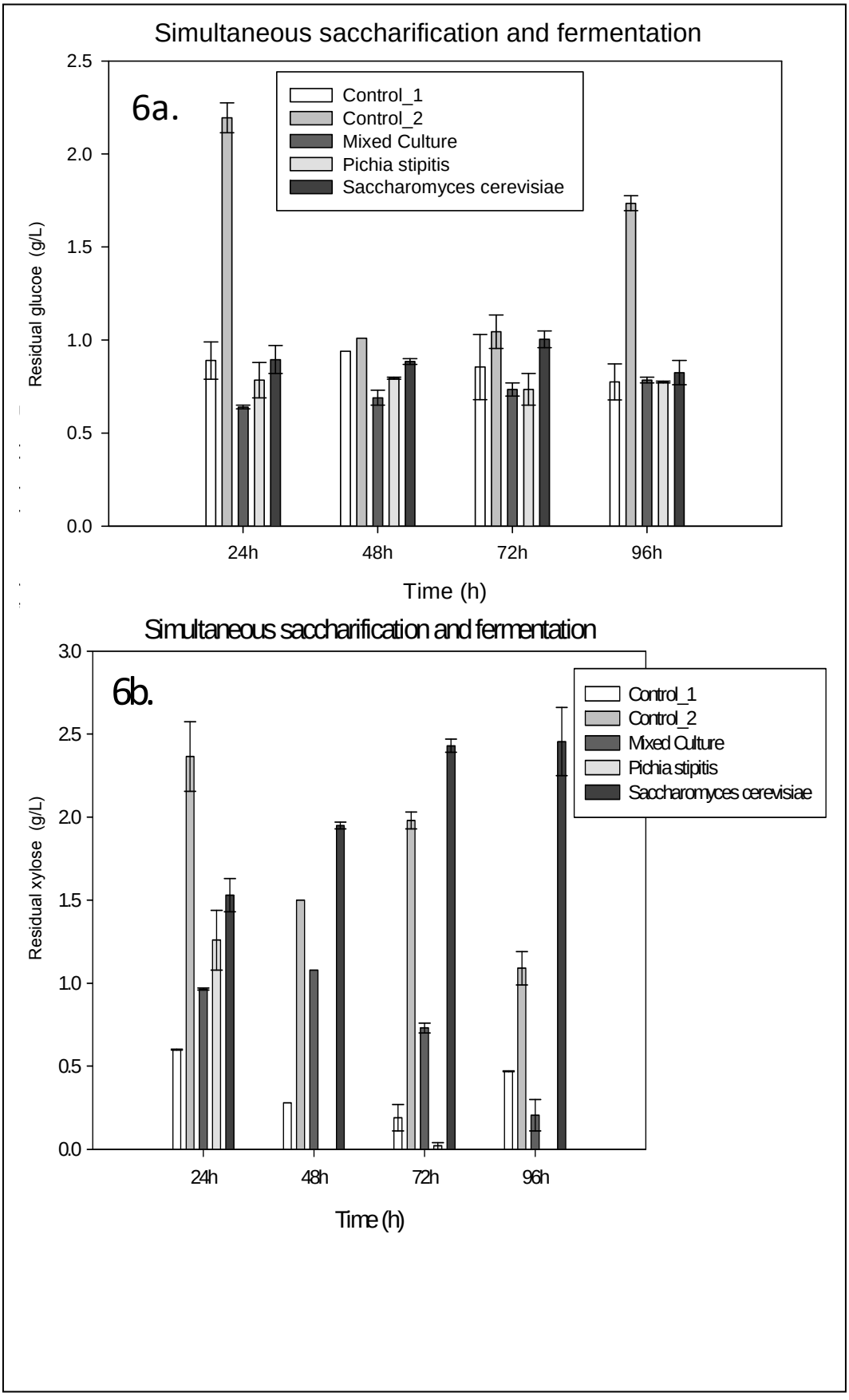


Fig. 5 Lime pretreatment and enzyme hydrolysis. a. Glucose yield b. xylose yield. Lower-case letters compare treatments with 50% crude enzyme hydrolysis and upper-case letters compare treatments with 100% crude enzyme. Treatments that do not share a letter are significantly different from others at P=0.05.


Simultaneous saccharification and fermentation
6a.
Control_1
Control_2
Mixed Culture
Pichia stipitis
Saccharomyces cerevisiae
Residual glucoe (g/L)
2.5
2.0
1.5
1.0
0.5
0.0
24h
48h
72h
96h
Time (h)
Simultaneous saccharification and fermentation
6b.
Control_1
Control_2
Mixed Culture
Pichia stipitis
Saccharomyces cerevisiae
Residual xylose (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24h
48h
72h
96h
Time (h)

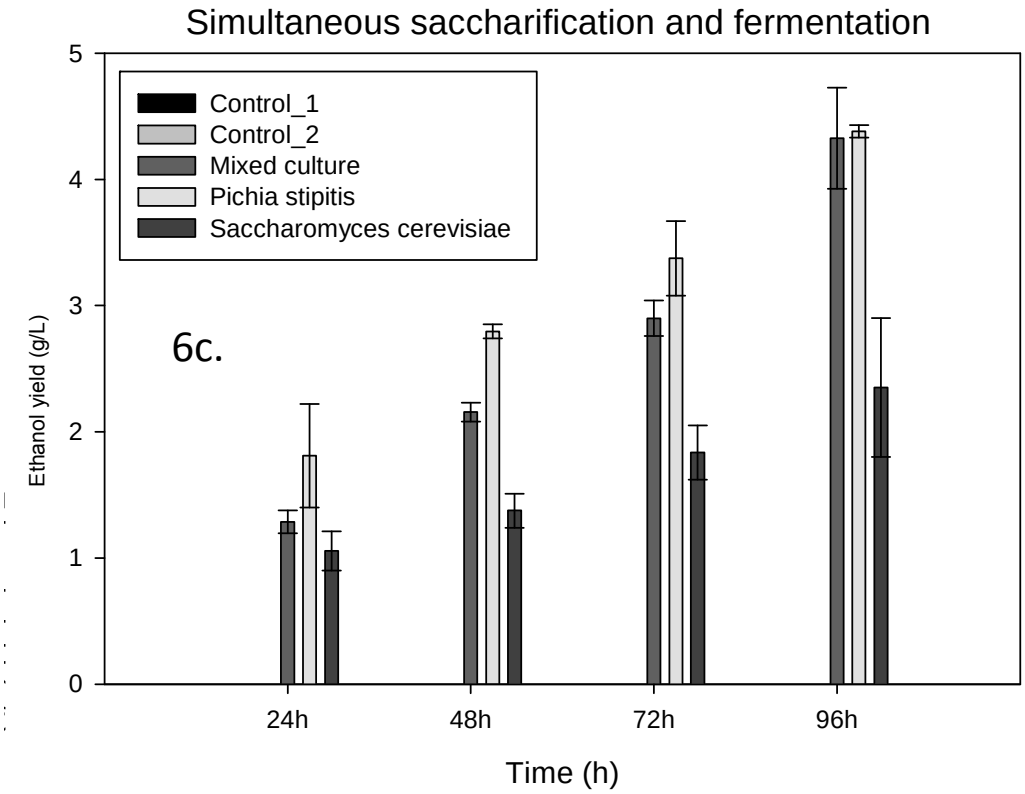


Fig. 6 Simultaneous saccharification and fermentation (SSF) using switchgrass pretreated with dilute sulfuric acid and hydrolyzed with 100% crude enzyme. a. Residual glucose b. Residual xylose and c. Ethanol yield at different time points. Control 1 is unpretreated switchgrass with 100% crude enzyme and control 2 is pretreated and hydrolyzed switchgrass

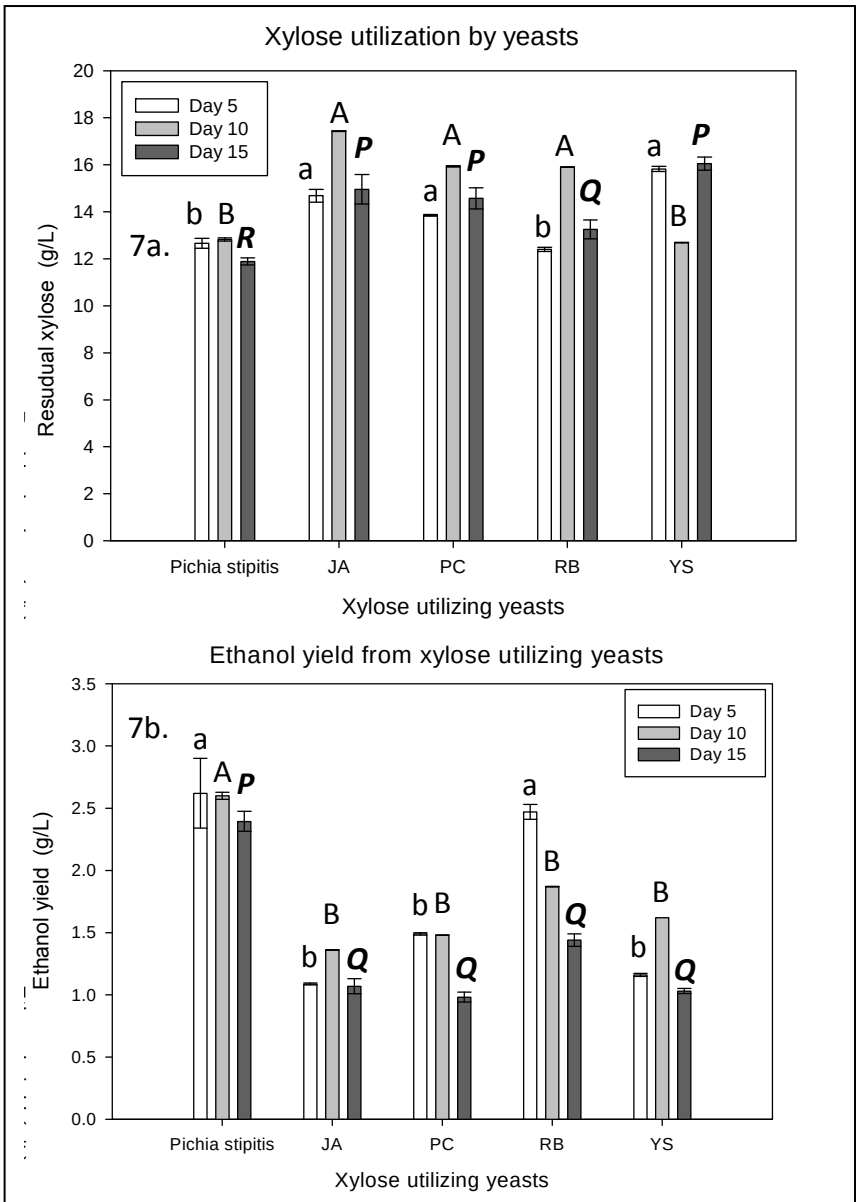


Fig. 7 Fermentation of xylose utilizing yeasts. a. Residual xylose b. Ethanol yield. Lower-case letters compare treatments on day 5, upper-case letters compare day 10 and upper-case bold italicized letters compare day 15 fermentation. Treatments within each time period that do not share a letter are significantly different from each other at P=0.05

Evaluation of other xylose utilizing microbial isolates for xylose conversion to ethanol in liquid medium.

Figure 8 presents ethanol levels in cultures of other xylose utilizing microbial isolates. However, further comparative studies revealed that these isolates did not produce more ethanol than our previous isolates reported in the third quarter.

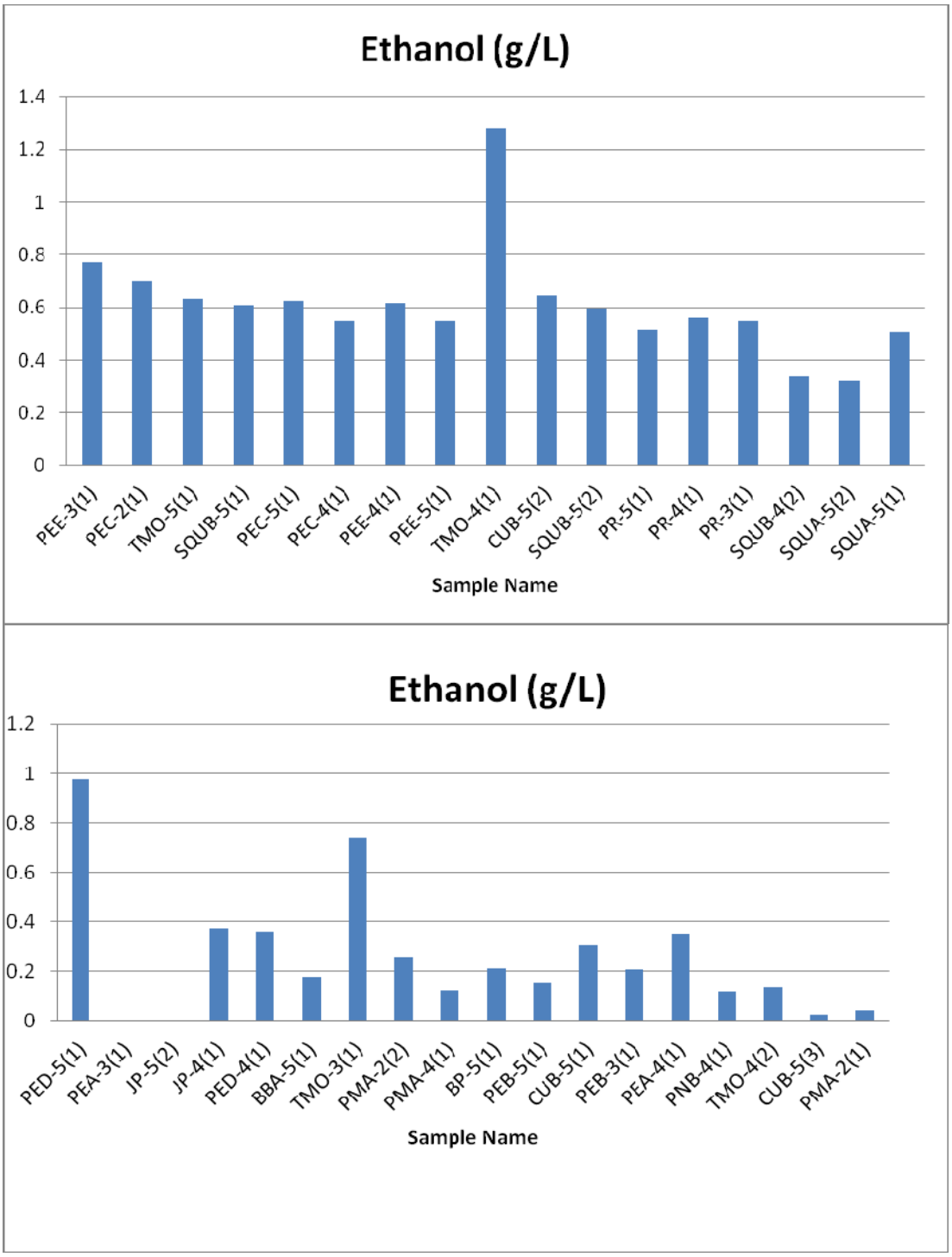


Figure 8: Ethanol level in cultures of other yeast isolates

Conclusions

Pretreatment and saccharification of switchgrass

Preliminary investigations revealed that 5, 10 or 10% crude enzyme were not sufficient to release high levels of sugar from the biomass. Further, 100% was found to be better than 50% enzyme for saccharification. From a practical viewpoint, sodium hydroxide was not evaluated further as it is a toxic chemical and its disposal can become an issue on the farm. Among three pretreatments namely dilute sulfuric acid, lime and ammonia, dilute sulfuric acid was found to be an ideal method. This method can be advantageous in that it can be recycled on the farm and used until it is saturated.

Simultaneous saccharification and fermentation

The glucose yield at 24h in the control 2 (pretreated switchgrass with saccharification) was 2.7g/L and theoretical ethanol yield should be 1.35g/L. Based on the glucose and xylose release in the control 2 at 24h, and the residual sugars in the yeast treatments, it is clear that mixed culture utilized 1.06g/L of glucose and 1.4g/L xylose, totaling 2.46g/L sugar and the theoretical ethanol yield should be 1.23g/L, whereas the actual yield was 1.28g/L. Similarly, *P. stipitis* consumed 1.9g/L glucose and 1.1g/L xylose, totaling 3.0g/L sugar and the theoretical ethanol yield should be 1.5g/L which is close to the actual yield of 1.81g/L. Finally, *S. cerevisiae* used 1.8g/L glucose (*S. cerevisiae* is not a xylose utilizing yeast), so the theoretical ethanol yield should be 0.9g/L, which is close to 1.05g/L.

The reduction in sugar content over time in control 2 is an indicator of microbial growth, possibly the growth of *Trichoderma* SG2 from the crude enzyme. It should be noted that the *Trichoderma* SG2 grown on soy hull for 4 days was mixed well and stored at -20^0C. At the time of SSF, a known amount of the crude enzyme sample was extracted with sterile water containing 2ppm lactrol. Although lactrol (virginiamycin) is an antibacterial, it is quite probable that the spores of *Trichoderma* SG 2 were able to grow to some extent and use the sugars for growth. It is also possible that the concentration of lactrol is not sufficient for complete suppression of bacteria in the biomass. We used 2ppm lactrol as it is the recommended dosage in corn ethanol production. Traditionally, sodium azide is used as an antimicrobial in cellulosic ethanol but is a pollutant of ground water. Keeping our objective of farm deployable microbial reactor for cellulosic ethanol in mind and also the possible utilization of the spent materials from this technology in animal feed, lactrol a USDA-approved antibacterial was used.

After 96h of SSF, *P. stipitis* and mixed culture of *P. stipitis* and *S. cerevisiae* showed similar ethanol production of 4.4g/L which was almost twice the yield in *S. cerevisiae* alone. In conclusion, *P. stipitis* is the yeast of choice for ethanol production under SSF from switchgrass that has been pretreated with 2% sulfuric acid and hydrolyzed with 100% crude enzyme of *Trichoderma* SG2. However, it should be noted that the highest sugar yield in control 2 is about 5g/L at 24h, whereas hydrolysis of pretreated biomass at 50 deg C has resulted in a maximum of 10g/L sugar after 72h which has the potential to yield higher ethanol than from SSF. Published reports suggest that ethanol yields from SSF and separate hydrolysis and fermentation (SHF) of

switchgrass are variable: Wyman et al (1992) have shown higher ethanol yield in SSF over SHF in acid treated switchgrass whereas Chung et al (2005) showed comparable ethanol yields in dilute acid pretreated switchgrass under SSF and SHF.

Finally, the sugar and ethanol yields from switchgrass using crude enzyme in this study are indeed remarkable compared to a recent SSF study by Sukumaran et al (2009) where they obtained 12% sugars and 25.56g/L ethanol after 24h from rice straw using *S. cerevisiae*. They used crude enzymes produced by *Trichoderma reesi* RUT C30 and *Aspergillus niger* MTCC 7956 on wheat bran by solid-state fermentation. But they were able to obtain higher levels of sugars due to concentration of the crude enzyme. In our experiments, we have used the raw crude enzyme without concentrating the enzymes. Purifying and concentrating the enzymes defeats our objective of farm deployable ethanol production and cannot be achieved practically on the farm.

Fermentation of xylose utilizing yeasts

The highest ethanol yield of 2.6g/L was seen in the control, *P. stipitis* fermentation which was similar to that from our isolate RB. Since the ethanol yields reached the peak on day 5 for most of the yeasts in this experiment and did not increase with time, it is best to stop fermentation by day 5 and recycle the process to achieve higher ethanol production. Our isolated xylose utilizing yeasts could not produce ethanol higher than that of the commonly used *P. stipitis* under the culture conditions used and will require optimization of fermentation conditions to determine their ethanol production efficiency.

Provisional patent filed

On October 13, 2011, a provisional patent titled ***Farm deployable bioreactor system for self-sufficient production of fuel ethanol using novel biomass degrading microbes*** was filed by the Auburn University Office of Technology Transfer. US Provisional Patent Application number 61/546,831. This patent is an initial protection of the idea of self-sufficient production in the farm.

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PART 4: Research Activities of FY-12 (October 1, 2011 to December 30, 2011).
Written by: Benedict Okeke and Ananda Nanjundaswamy

Introduction

The objectives of this study were 1) employ DNA methods to characterize microbial isolates to support selection of microbial catalysts for development of lignocellulose ethanol bioreactor system, 2) employ response surface methodology (RSM) for submerged production of enzymes, 3) to evaluate media components for solid-state fermentation (SSF) for enzyme production, 4) evaluate optimal fermentation period for enzyme production from switchgrass in solid-state, 5) initiate design and evaluation of lab-scale 'farm-deployable' bioreactor starting with scale-up of enzyme production in lab model bioreactor 6) based on all the data available thus far, initiate manuscript preparation.

In our third quarter research, submerged fermentation of screening medium (paper powder:switchgrass 1:1) for enzyme production was optimized using response surface methodology (RSM). The model predicted the enzyme production from using optimized media components. The enzyme production was validated using optimized media components.

In our third quarter report, enzyme production from SSF of nine substrates by *Trichoderma* SG2 was outlined and following statistical analyses it was found that soy hull was the best substrate for enzyme production. However, after careful considerations, switchgrass as a substrate for SSF production of enzymes was investigated since switchgrass is locally grown and easily available. Keeping in mind the practical considerations of 'farm-deployable' bioreactor and using easily available resources on the farm, switchgrass (with or without pretreatment), tap water (boiled or autoclaved), nitrogen source (urea or yeast extract peptone mixture) were evaluated as SSF media components for enzyme production. Further, the duration of fermentation (5d, 10d or 15d) for maximum enzyme production from switchgrass was evaluated.

Before setting up a farm-scale bioreactor, preliminary investigations into a lab-scale 'farm-deployable' bioreactor was developed using a five gallon brewing vessel. The lab-scale bioreactor was designed to test 1) enzyme production using switchgrass submerged fermentation, 2) simultaneous saccharification and fermentation, 3) separate hydrolysis and fermentation and 4) the best model will be tested with mixed culture fermentation of *Saccharomyces cerevisiae* and *Pichia stipitis*.

Materials and Methods

Molecular characterization of microbial isolates

DNA extraction

Media for cultivation of filamentous fungal biomass consisted of potato dextrose agar 39 g/L (potato starch 4.0 g/L; dextrose 20.0 g/L; and agar 15.0 g/L). Media for cultivation of yeast biomass consisted of the following: yeast extract 10 g/L; peptone 20 g/L; xylose 10 g/L; chloramphenicol 0.075 g/L, and agar 20 g/L. Yeasts were grown at 30^{o}C for 3 days? Molds were grown at 30^{o}C for 4 days. DNA was extracted from yeasts and molds using UltraClean^{TM} Soil DNA isolation kit (MOBIO, USA)

according to the manufacturer's instructions. Briefly, yeast cells and mycelia suspensions in water were obtained from agar plates. About 1 mL of each suspension was transferred to a beaded solution tube from the Mo Bio DNA isolation kit. Sixty micro-liters of SDS solution (Mo Bio Solution S1) was added to the beaded solution tube, and was vortexed briefly and 200 µL of inhibitor removal solution (Mo Bio Solution S2) was added to the tube and vortexed for ten minutes. After vortexing, the tube was centrifuged for thirty seconds at 10,000 x g. The supernatant was transferred to a two mL collection tube provided by Mo Bio, and 250 µL of solution S2 was added to the tube. The tube was centrifuged for one minute at 10,000 x g. The supernatant was transferred to another 2 mL supernatant tube and 1.3 mL of solution S3 was added to this tube. This was then centrifuged for 1 minute at 10,000 x g, and 700 µL of the supernatant was added to the spin filter. The flow through was discarded and the remaining supernatant from the 2 mL tube was added to the spin filter and centrifuged. This process was repeated until all the supernatant from the 2 mL tube passed through the spin filter. About 300 µL of ethanol based wash solution (Mo Bio solution S4) was added to the spin filter and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the spin filter was placed into a new 2 mL collection tube. Thereafter, 50 µL of sterile ethanol buffer (Mo Bio solution S5) was added to the spin filter, and this was centrifuged for 30 seconds at 10,000 x g. The spin filter was discarded and the DNA was preserved by freezing.

DNA was extracted from xylose utilizing microorganisms shortlisted by microscopy to be possibly bacteria as follows. The isolates were grown by streaking on tryptic soy agar (TSA) and incubated at 30°C for 24 h for evaluation of culture purity. Colonies of the bacterium were suspended in nuclease-free water (Promega, Madison, WI). Cells were recovered by centrifugation. DNA was extracted using Promega wizard genomic DNA purification kit (Promega, Madison, WI) with slight modification. Briefly, cells were re-suspended in 600 µL of nucleic acid lysis solution, incubated at 80°C for 5 min and allowed to cool to room temperature. RNase solution (3 µL) was added and incubated at 37°C for 20 min. Protein precipitation solution (200 µL) was added and the tubes were incubated on ice for 5 min. Following centrifugation, the supernatant was transferred to a tube and ice cold 95% ethanol was added. The precipitate was recovered by centrifugation. The pellet was washed with 70% ethanol at ambient temperature and re-suspended in rehydration solution.

Polymerase Chain Reaction Amplification (PCR) of DNA

Conserved primer sequences, LR0R of *Saccharomyces cerevisiae* positions 26-42 (5'-ACCCGCTGAACTTAAGC-3') and LR5 of *Saccharomyces cerevisiae* positions 964-948 (5'-TCCTGAGGGAAACTTCG-3') were employed for PCR amplification of a region of the large subunit (LSU) ribosomal RNA of yeasts and molds. Whereas, conserved primer sequences, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were employed for PCR amplification of a region of the intergenic spacer region (ITS) of ribosomal RNA of yeasts and molds.

Bacterial universal primers corresponding to *Escherichia coli* positions 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used for PCR amplification of the 16S ribosomal RNA gene (Lane, 1991). The PCR reaction mixture consisted of 25 µL of PCR master mix (Promega, Madison,WI), genomic DNA template (2 µL), primer 27f (5 µL=25 pmol), and primer 1492r (5 µL=25 pmol) and made up to 50 µL final

volume with nuclease free water. The 16S rRNA gene was amplified using a 35-cycle PCR (initial denaturation, 95 °C for 5 min; subsequent denaturation, 95 °C for 0.5 min; annealing temperature, 50 °C for 1 min; extension temperature, 72 °C for 1 min and final extension, 72 °C for 5 min). The PCR amplification products were analyzed by electrophoresis on a 1% agarose gel. E.Z.N.A. cycle-pure kit (Omegabiotek, Norcross GA) was used to remove primers, salts, and unincorporated dNTPs according to the manufacturer's instructions.

DNA sequencing and similarity analysis

Cycle sequencing of DNA templates was performed using BigDye terminator kit (Applied Biosystems, Foster City, CA) with each corresponding PCR primer as well as primer 519r (5′-GWATTACCGCGGCKGCTG-3′) for bacteria together with mixtures of dNTPs, fluorescently-labeled dideoxynucleotide triphosphates at the Institute of Integrative Genome Biology, UC Riverside, CA. GenBank nucleotide blast was used for similarity searches.

Optimized medium from RSM study

The optimized screening medium contained 0.62% paper powder, 0.96% switchgrass, 0.06% yeast extract, 0.14% peptone and all other ingredients remained the same as in unoptimized medium (0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution).

Acid Pretreatment

For acid treatment H_2SO_4 was used (Chung et al. 2005). A 2% solution of H_2SO_4 was prepared using de-ionized water. Approximately 10g of switchgrass was weighed into a 250ml flask and 90ml of 2% H_2SO_4 was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 121^0 C for 60min. After autoclaving flasks were carefully removed and strained using a muslin cloth to remove liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during acid hydrolysis. The solid portion (pretreated switchgrass) was washed several times to remove acid. The complete removal of the acid was confirmed by suspending a small portion of the sample in water and pH measured. Pretreated samples were air dried at room temperature for 24-48h.

SSF media for enzyme production

About 10g of switchgrass with or without acid pretreatment was adjusted with tap water to contain 70% moisture in a petri dish. Tap water was boiled or sterilized by autoclaving at 121° C for 15 min. Nitrogen source included 0.1% urea (normally used in enzyme production media) or 0.1% yeast extract and 0.05% peptone. Totally there were 12 treatments, and each treatment received two replicates. The plates were incubated at 30 °C for 5d.

Further, based on the best media components for maximum enzyme production, switchgrass SSF medium without pretreatment, boiled water and 0.1% urea was used for enzyme production for 5d, 10d and 15d.

Lab-scale 'farm-deployable' bioreactor

Materials used: 5 gallon borosilicate carboy (lab-vessel) for brewing, silicone heating blanket (24"X36") (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), temperature probe (Cole-Parmer), Digi-Sense Potential Integrative Detector (PID)—temperature controller (Cole-Parmer), magnetic stirrer (Model 1000, VWR International, Radnor, PA, USA), silicone tubing (VWR), air pump, peristaltic pump (VWR).

Submerged fermentation medium: Previously used screening medium was used for this study but the paper powder was substituted with switchgrass since mixing of the medium in large-scale was problematic. A liter of the medium contained 10.0g of pulverized switch grass, 1.0g of peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution.

Preliminary set up of laboratory model: Approximately 10L of screening medium was filled into a 5 gallon lab vessel. The unit was fitted with silicone tubing for air inlet, air outlet, inoculation and sample collection. Air inlet was fitted with 25mm 0.25um air-filter. A 2" magnet was dipped into the vessel before sterilization.

Preliminary operation of the lab model: The vessel was autoclaved at 121°C for 30 min. Upon autoclaving the vessel was allowed to cool to room temperature. An alcohol-sterilized temperature probe was inserted into the vessel. The vessel was moved onto a high capacity magnetic stirrer. The stirrer was slowly turned on and was slowly increased to1600 rpm. Connection from the air pump was connected to the air filter. The temperature probe was connected to the controller and the heating blanket was wrapped around the vessel (as shown in the figure 3). The blanket power cord was plugged into the temperature controller. Desired temperature of 30°C was set in the controller. Approximately 100 ml of 48h well grown inoculum of *Trichoderma* SG2 was transferred into the vessel using peristaltic pump using dedicated inoculation. Air pump was turned on to keep the medium under aerobic condition. Samples were harvested on days 4, 5, 6 and 8, and enzyme analysis was carried out.

Lipid production by red yeast fermentation of peanut meal for microbial biodiesel

Several yeasts were isolated in this study (Okeke et al. 2011). Red yeasts or carotenogenic yeasts isolated from this study are being characterized for lipid production in waste media such as peanut meal. Lipids extracted from the yeasts can be transesterified with lignocellulose ethanol to produce biodiesel.

Statistical analyses

Data were analyzed statistically using SAS (version 9.1.4). Analysis of Variance (ANOVA) was carried out to compare treatments using PROC ANOVA. Pairwise-comparison was carried out using Tukey adjustment and significance was set at P=0.05.

Results

Molecular Characterization of isolates

Table 1 presents molecular characterization of yeast isolates by ribosomal RNA gene sequence analysis. Ribosomal RNA gene sequence revealed that yeast isolate YS28 was most identical (99%) to *Debaryomyces pseudopolymorphus (*EF198011.1). Yeast isolate PCH-3 was most identical (99%) to *Meyerozyma (Pichia) guilliermondii (*HQ857743.1; JN940622.1). Similarly PCH-4 was most identical to *Pichia guilliermondii (*AY497675.1). Red yeast or carotenogenic yeasts (R1 – R8) were also characterized (Table 1). DNA sequences obtained with different primers showed that the strains are most identical to *Rhodotorula* species especially *Rhodotorula mucilaginosa*. Yeast isolates Y1 –Y3 were most identical to *Meyerozyma guilliermondii.*

Molecular characterization of filamentous and related fungi is presented in Table 2. Fungal isolates SG2 and SG4 were most identical (99%) to *Hypocrea nigricans* (99% identity) *and Trichoderma harzianum* (99% identity), respectively. FS17A was 100% identical to *Hypocrea nigricans* (JN941469.1). FS3 was 99% identical to *Fusarium oxysporum* (JN232163.1; EF590327.1). FS18 was most identical (93%) to *Fusarium proliferatum* (FJ890385.1). FS25 was most identical (99%) to *Hypocrea nigricans.* FS23A displayed 100% similarity to *Hypocrea nigricans (*JN941469.1). Isolate SBD25B was 99% identical to *Debaryomyces pseudopolymorphus (*EF198011.1). TMO-4 was most identical *(*99%) to *Issatchenkia orientalis (*EF550222.1). PEE-3 was 96% identical to *Candida quercitrusa (*AM158924.1). Isolate SBD-4 was most identical (95%) to *Debaryomyces hansenii* (JN938932.1). PED-5 was 99% identical to *Zygoascus meyerae (*DQ438189.1). RBP-3 was 99% identical to *Eurotium amstelodami (*JN021532.1). FS5A showed 99% identity to *Trichoderma amazonicum (*JN939814.1) and *Hypocrea lutea (*JN941457.1*).* FS22A and FS27 were 100% and 99% identical to *Penicillium janthinellum (*JN246046.1) and *Penicillium sp. BCC 17468 (*GU809209.1), respectively. PEC-4 rRNA gene sequence displayed no significant similarity and was only 80% identical to *Fusarium oxysporum (*EF590327.1). SBD- 21B displayed 99% identity to *Candida tropicalis.*

Ribosomal RNA gene sequence analysis of 5 xylose utilizing bacteria is presented in Table 3. RBP-5 displayed 99% similarity to *Enterobacter asburiae* (JN033555.1) Isolate JAP-4 is 99% identical to *Pseudomonas plecoglossicida* (EU594553.1). JAP-3 showed 100% similarity to *Pseudomonas sp. SL6* (HQ283480.1). TMO-3 was 99% identical to *Acinetobacter calcoaceticus (*FJ816053.1). Whereas, isolate CRP-5 showed to *Enterobacter sp (*HM459842.1*).*

Validation of RSM for enzyme production by submerged fermentation.

The enzyme activities for cellulase, xylanase, beta-glucosidase, and beta-xylosidase obtained were upon validation 10.86 (±0.74 SE), 35.21 (±0.05), 6.07 (±0.32) and 5.94 (±0.08) U/ml respectively and were found to be comparable to the predicted values (Fig. 1).

Enzyme production from switchgrass SSF

Table 4 provides the ANOVA for effects of media components on enzyme production. Cellulase production was not affected by sterilization or nitrogen source but was affected by pretreatment of substrate namely switchgrass. Acid-pretreated switchgrass

was found to provide significantly higher cellulase activity than untreated switchgrass (Table 4).

Xylanase enzyme production was influenced both by pretreatment and sterilization, whereas nitrogen source had no effect (Table 4). Untreated switchgrass yielded significantly more enzyme compared to acid-treated substrate, and sterilized water yielded higher enzyme than boiled water.

Beta-glucosidase production was affected by all three factors: untreated switchgrass yielded significantly higher enzyme than acid-treated substrate, boiled water was better than sterilized water and urea was the best nitrogen source followed by yeast extract-peptone mixture which was better than no supplementation of nitrogen source.

Pretreatment and sterilization of water had no effect on beta-xylosidase whereas nitrogen source significantly affected the enzyme production. Yeast extract-peptone mixture and urea resulted in similar enzyme production and both were greater than that when no nitrogen was supplemented.

Enzyme production in switchgrass SSF on day 5, 10 and 15 were evaluated (Fig 2). The ANOVA for effect of time of fermentation on enzyme production: cellulase F=19.77 and *P=0.0187*; xylanase F=25.73 and *P=0.0129*; beta-glucosidase F=6.41 and P=0.0825; beta-xylosidase F=3.34 and P=0.1727. The highest cellulase and xylanase production was seen on day 15 and was significantly greater on day 10 and 5. Enzyme production on day 5 and 10 did not vary significantly. The production of beta-glucosidase and beta-xylosidase enzymes did not vary over time and the best production was on day 10 and day 5 respectively.

Enzyme Production Process Scale-Up for 'farm-deployable' bioreactor

A 5-gallon lab-scale 'farm-deployable' bioreactor was designed and developed for enzyme production by submerged fermentation (Fig. 3). Enzyme production on day 4, 5, 6 and 8 of fermentation was recorded (Fig. 4). Day 8 yielded the highest activities for all four enzymes: 1.54 U/ml cellulase, 26.51 U/ml xylanase, 0.28 and 0.25 U/ml beta-glucosidase and xylosidase respectively.

Lipid production by red yeast fermentation

This study used yeast isolates in peanut meal fermentation for lipid production. Preliminary studies indicated substantial accumulation of lipids by some red yeast isolates. Results suggest that there is potential for conversion of the yeast lipids to biodiesel by esterification with lignocellulose ethanol.

Table 1:Characterization of yeast isolates based on ribosomal RNA gene sequence analysis.

Isolate code	Sequencing Primer	Number of nucleotides	Closest GenBank match	Closest match accession number	% identity
YS28	LROR	600	*Debaryomyces pseudopolymorphus*	EF198011.1	91
	ITS1	530	*Debaryomyces hansenii*	FR870476.1	81
	ITS4	1110	*Debaryomyces pseudopolymorphus*	EF198011.1	99
	LR5	900	*Debaryomyces hansenii*	JN938932.1	97
PCH-3	LROR	650	*Pichia sp.*	FN999999.1	98
	ITS1	560	*Meyerozyma guilliermondii*	HQ857743.1	99
	LR5	900	*Meyerozyma guilliermondii*	JN940622.1	99
PCH-4	LROR	520	*Pichia guilliermondii*	AY497675.1	99
R1	LROR	650	*Rhodotorula sp.*	EF585197.1	99
	ITS1	580	*Rhodotorula glutinis*	HQ670677.1	92
	ITS4	574	*Rhodotorula mucilaginosa*	FN428885.1	99
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	98
R2	LROR	850	*Rhodotorula mucilaginosa*	EF585187.1	95
	ITS1	800	*Sporobolomyces sp. AL-V*	JN255485.1	96
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	97
R3	LROR	550	*Rhodotorula mucilaginosa*	FN428899.1	99
	ITS1	820	*Rhodotorula glutinis*	HQ670677.1	94
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	94
R4	LROR	650	*Rhodotorula sp. CRUB 1484*	EF585197.1	99
	ITS1	580 830	*Rhodotorula glutinis* *Rhodotorula mucilaginosa*	HQ670677.1 FN428885.1	94 94
	ITS4	572	*Rhodotorula mucilaginosa*	FN428885.1	99
	LR5	900	*Rhodotorula mucilaginosa*	DQ832198.1	98
R5	LROR	650	*Rhodotorula sp. CRUB 1484*	EF585197.1	99
	ITS1	580	*Rhodotorula glutinis* *Rhodotorula mucilaginosa*	HQ670677.1 FN428885.1	98 98
	ITS4	574	*Rhodotorula mucilaginosa*	FN428885.1	99
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	98
R6	LROR	550	*Rhodotorula sp. Z100(C)*	FJ795016.1	99
	ITS1	580 800	*Rhodotorula mucilaginosa* *Rhodotorula mucilaginosa*	EU871490.1 JN209850.1	97 97
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	99

Table 1 continued: Characterization of yeast isolates based on ribosomal RNA gene sequence analysis

R7	LROR	650	*Rhodotorula sp. CRUB 1484*	EF585197.1	99
	ITS1	580	*Rhodotorula sp. BCRC*	GU646862.1	94
	ITS4	571	*Rhodotorula mucilaginosa*	FN428885.1	96
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	98
R8	LROR	550	*Rhodotorula mucilaginosa*	FN428899.1	99
	ITS1	580 850	*Rhodotorula sp. BCRC* *Rhodotorula glutinis*	GU646862.1 HQ670677.1	94 96
	LR5	700	*Rhodotorula mucilaginosa*	DQ832198.1	98
Y1	LR5	900	*Meyerozyma guilliermondii*	JN940619.1	97
Y2	LR5	750	*Meyerozyma guilliermondii*	JN940622.1	85
Y3	LR5	750	*Meyerozyma guilliermondii*	JN940622.1	85

Table 2: Characterization of filamentous fungi based on ribosomal RNA gene sequence analysis

Isolate code	Sequencing primer	Number of nucleotides	Genbank match	Genbank match accession number	% Identity
SG2	ITS4	829	*Hypocrea lixii*	FR872742.1	93
	LR5	700	*Hypocrea nigricans*	JN941469.1	99
SG4	ITS4	990	*Hypocrea lixii*	FR872742.1	94
	LR5	900	*Trichoderma harzianum*	JN939829.1	99
FS 17A	ITS4	810	*Hypocrea lixii genomic*	FR872742.1	89
	LR5	510	*Hypocrea nigricans*	JN941469.1	100
FS 3	ITS4	510	*Fusarium oxysporum*	JN232163.1	99
	LR5	510	*Fusarium oxysporum*	EF590327.1	99
FS 18	ITS4	510	*Hypocrea nigricans*	JN941469.1	76
	LR5	520	*Fusarium proliferatum*	FJ890385.1	93
FS 25	ITS4	530	*Hypocrea nigricans strain*	JN941469.1	85
	LR5	550	*Hypocrea nigricans strain*	JN941469.1	99
FS 23 A	ITS4	500	*Rhodotorula mucilaginosa*	DQ832198.1	77
	LR5	520	*Hypocrea nigricans*	JN941469.1	100
SBD 25B	ITS4	839	*Debaryomyces pseudopolymorphus*	EF198011.1	99
	LR5	550	*Debaryomyces hansenii*	JN938932.1	96
TMO-4	ITS4	470	*Issatchenkia sp. YF04a*	DQ667976.1	99
	LR5	610	*Issatchenkia orientalis*	EF550222.1	99
PEE-3	ITS4	570	*Candida quercitrusa*	AM158924.1	96
	LR5	520	*Debaryomyces hansenii*	JN938932.1	93
SBD-4	ITS4	550	*Penicillium sp. BCC 17468*	GU809209.1	88
	LR5	530	*Debaryomyces hansenii*	JN938932.1	95
PED-5	ITS4	870	*Zygoascus meyerae*	HM450997.1	92
	LR5	510	*Zygoascus meyerae*	DQ438189.1	96
	LR5	890	*Zygoascus meyerae*	DQ438189.1	99

Table 2 continued: Characterization of filamentous fungi based on ribosomal RNA gene sequence analysis

RBP-3	ITS4	520	*Aspergillus niger strain*	JN802255.1	87
	LR5	520	*Eurotium amstelodami*	JN021532.1	99
FS 5A	LR5	880	*Trichoderma amazonicum* *Hypocrea lutea*	JN939814.1 JN941457.1	99 99
FS 22A	LR5	750	*Penicillium janthinellum*	JN246046.1	100
FS 27	LR5	520	*Penicillium sp. BCC 17468*	GU809209.1	99
	LR5	610	*Penicillium sp. BCC 17468*	GU809209.1	99
PEC-4	LR5	510	*Fusarium oxysporum*	EF590327.1	80
SBD-21B	LR5	700	*Candida tropicalis*	EF192215.1	98
	LR5	650	*Candida tropicalis*	JN940626.1	99

Table 3: Characterization of xylose fermenting bacteria based on ribosomal RNA gene sequence analysis

Isolate code	**Sequencing primer**	**Number of nucleotides**	**Genbank match**	**Genbank match accession number**	**% Identity**
RBP-5	519r	482	*Enterobacter asburiae*	JN033555.1	99
	27f	650	*Enterobacter asburiae*	JN033555.1	98
JAP-4	519r	480	*Bacterium enrichment culture clone ALO1*	JF688587.1	99
	27f	650	*Pseudomonas plecoglossicida*	EU594553.1	99
JAP-3	519r	480	*Pseudomonas sp 01WB03.1-24*	FM161432.1	99
	27f	650	*Pseudomonas sp. SL6*	HQ283480.1	100
TMO-3	519r	480	*Acinetobacter calcoaceticus*	FJ816053.1	99
	27f	650	*Acinetobacter calcoaceticus*	FJ816053.1	99
CRP-5	519r	498	*Enterobacter sp.*	JF920022.1	99
	27f	650	*Enterobacter sp.*	HM459842.1	99

Table 4: ANOVA

Enzymes/Sugar	**Effects**	**F**	***P***	**Notes**
Cellulase				
	Pretreatment	9.91	*0.0093*	Acid>None
	Sterilization	2.34	0.1543	
	Nitrogen Source	1.41	0.2851	
Xylanase				
	Pretreatment	16.99	*0.0012*	None>Acid
	Sterilization	6.29	*0.0134*	Autoclave>Boil
	Nitrogen Source	6.01	0.9958	
Beta-glucosidase				
	Pretreatment	46.24	*<0.0001*	None>Acid
	Sterilization	10.02	*<0.009*	Boil>Autoclave
	Nitrogen Source	21.35	*<0.0002*	Urea>YEPP>None
Beta-xylosidase				
	Pretreatment	3.93	0.0728	
	Sterilization	4.4	0.0599	
	Nitrogen Source	13.52	*0.011*	YEPP=Urea>None
Glucose				
	Pretreatment	133.55	*<0.0001*	Acid>None
	Sterilization	1.32	0.2755	
	Nitrogen Source	3.41	0.0704	
Xylose				
	Pretreatment	161.99	*<0.0001*	Acid>None
	Sterilization	6.29	*0.0291*	Autoclave>Boil
	Nitrogen Source	16.01	*0.0173*	Urea>None>YEPP

Pretreatment: Acid-treated, none
Sterilization of tap water used: autoclaved, boiled
Nitrogen source added: urea, yeast extract-peptone mixture

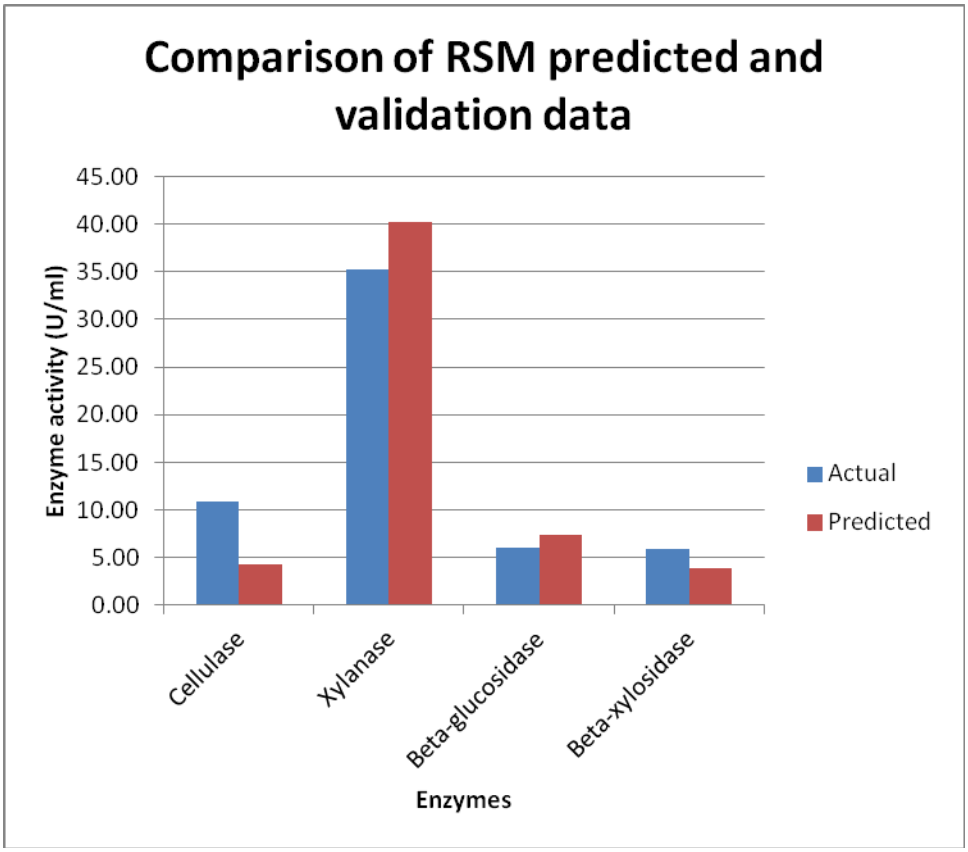


Figure 1. Comparison of enzyme activities in submerged fermentation of RSM predicted and validation experiments.

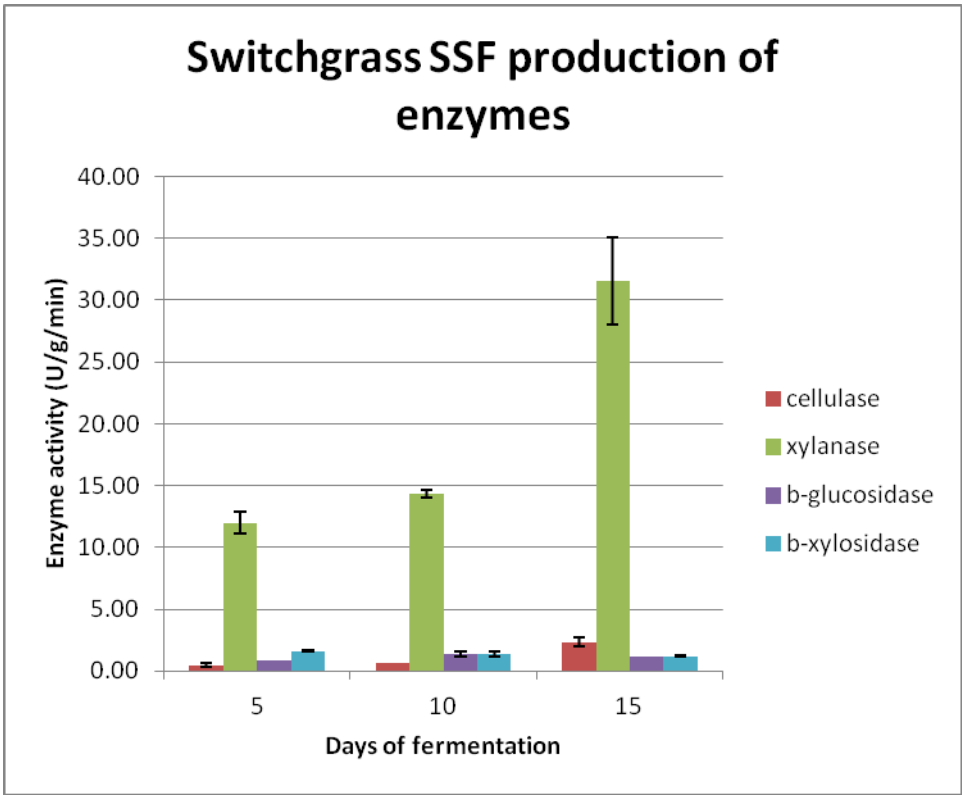


Figure 2. Production of enzymes in switchgrass SSF on days 5, 10 and 15.

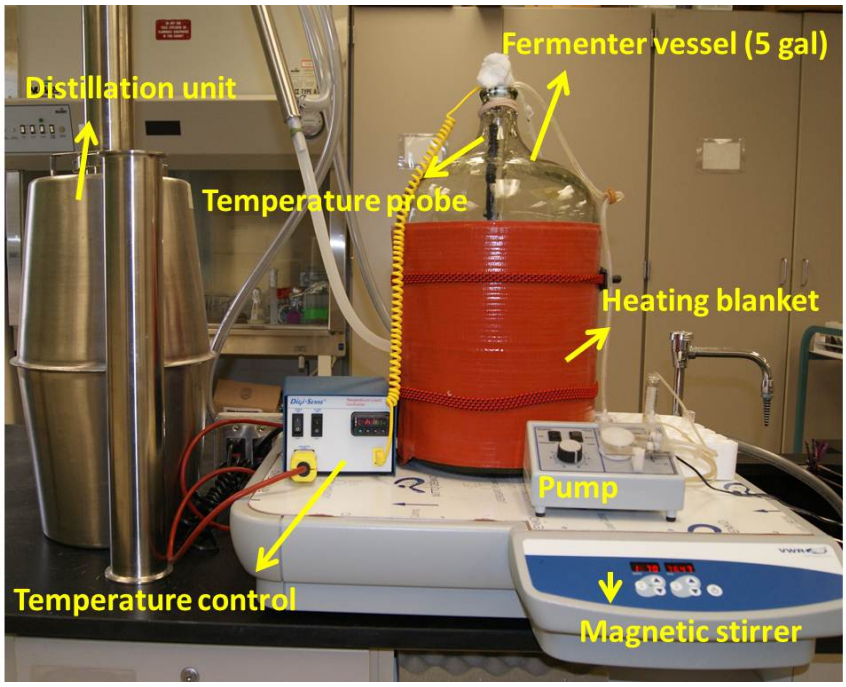
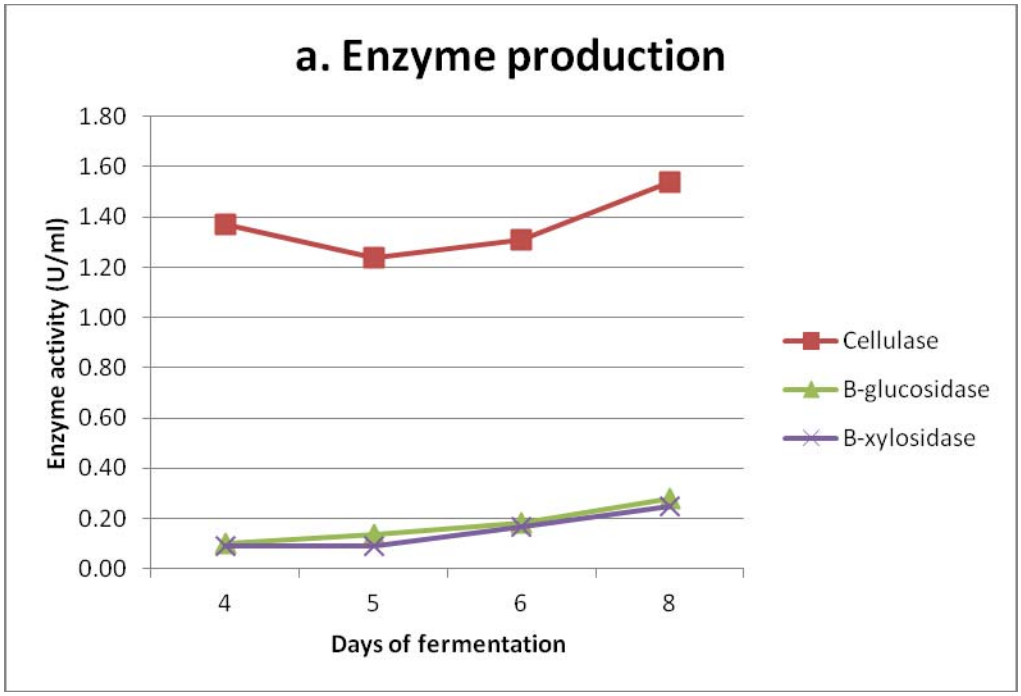


Figure 3. Scale-up enzyme production in laboratory bioreactor.

Figure 4



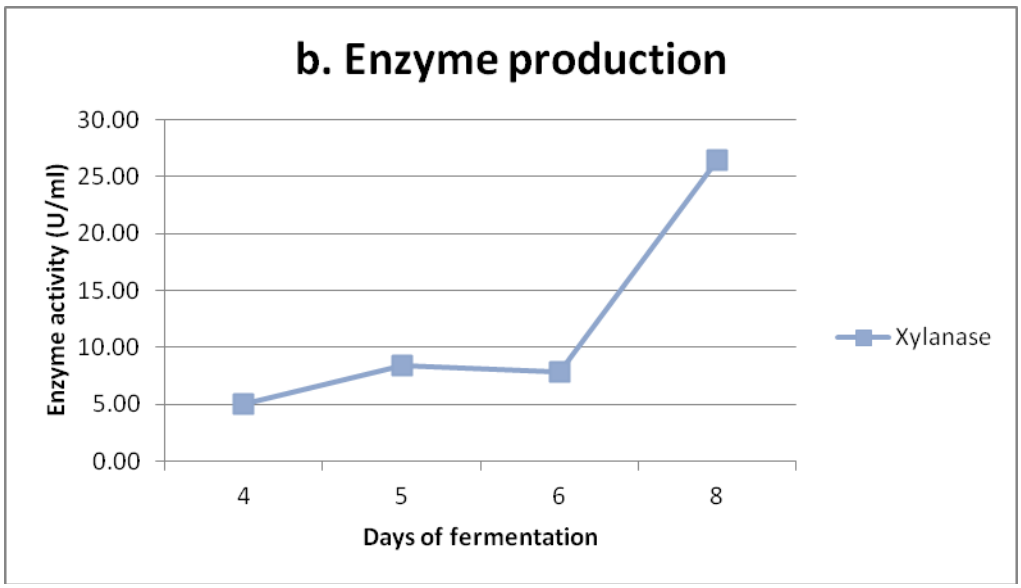


Figure 4. Enzyme production in submerged fermentation of switchgrass in laboratory bioreactor.

Conclusions

Selection of lignocellulose degrading fungi and xylose fermenting yeasts

Based on screening and characterization of organisms, four fungi, namely *Trichoderma* (*Hypocerea*) sp. SG2, *Trichoderma* (*Hypocerea*), *Fusarium oxysporum* FS22A, *Trichoderma amazonicum (Hypocrea lutea)* FS5A were selected as potential catalysts for farm deployable bioreactor development. Two xylose fermenting yeast, *Debaryomyces pseudopolymorphus* YS28 and *Meyerozyma guilliermondii* (*Pichia* sp.) were selected as potential catalysts for fermentation of lignocellulose hydrolysate. Two xylose fermenting bacteria identified by 16S rRNA sequencing were also selected as potential catalysts for fermentation of lignocellulose hydrolysate.

Validation of RSM for enzyme production by submerged fermentation

The predicted enzyme activities from submerged fermentation using screening medium from RSM and the validation enzyme activities were a close match for most enzymes. However, the validation activities for cellulase and beta-xylosidase were greater than predicted values, and the opposite was true for xylanase and beta-glucosidase. Cellulase production in validation resulted in 60% greater activity than predicted value, whereas xylanase showed 12% reduction in activity. The differences between predicted and validation results were negligible for beta-glucosidase and beta-xylosidase.

Solid-state fermentation of switchgrass for enzyme production

This study has shown that for best enzyme production in SSF, untreated switchgrass mixed with boiled water to maintain moisture and 0.1% urea as nitrogen source are ideal for overall enzyme production on the farm. A study by Ghori et al (2011) also reported that urea was superior to Ammonium sulfate as nitrogen source in cellulase production. Further, Maeda et. al. (2010) reported that urea and yeast extract combination was best for cellulase production. Pretreatment with 2% sulfuric acid, use of steam for autoclaving at 121°C for 30 min or expensive nitrogen sources like yeast extract and peptone can be avoided which are of practical value when using a 'farm-deployable' bioreactor on the farm. Urea is an inexpensive and commonly used fertilizer and easily available on the farm. However, the maximum cellulase and xylanase production were found to be on day 15, whereas day 10 and 5 were best for beta glucosidase and beta-xylosidase respectively. Overall, from practical stand point, it is best to carry out the fermentation for 10 days and harvest all enzymes on day 10.

Lab-scale 'farm-deployable' bioreactor

A working laboratory model of the 'farm-deployable' bioreactor has been designed and process optimization is underway. In the next quarter, we aim to use this optimized lab-scale model for optimal enzyme production, saccharification, and simultaneous saccharification and fermentation.

Presentations

1. A. Nanjundaswamy, R. Hall, B. C. Okeke* (2012). Lipid production by red yeast fermentation of peanut meal for microbial biodiesel. This was submitted to the

American Society of Microbiology (ASM) General Meeting to be held at ASM 2012, June 16-19, San Francisco, USA.

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1. Chung YC, Bakalinsky A, Penner MH (2005). Enzymatic saccharification and fermentation of xylose-optimized dilute acid-treated lignocellulosics. Applied Biochemistry and Biotechnology 121:947-961.

2. Ghori M., Ahme S., Malana M. A. Jamil A. (2011). Corn stover-enhanced cellulase production by *Aspergillus niger* NRRL 56 Afr. J. Biotechnol. 10(31), pp. 5878-588.

3. http://www.biology.duke.edu/fungi/mycolab/primers.htm: Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA. Vilgalys lab, Duke University. Accessed (October 2012).

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5. Maeda R N, da Silva M M, Santa Anna L M, Pereira N Jr (2010). Nitrogen source optimization for cellulase production by Penicillium funiculosum, using a sequential experimental design methodology and the desirability function. Appl. Biochem. Biotechnol. 161(1-8):411-22

6. Okeke BC., Deravi, Y., Prescott, A., Bishop, J., Sawyer, L., Peaks, S., Nanjundaswamy, A. and Hall, R (2011). Screening of xylose-utilizing yeasts from soil-decaying biomass composite samples and fruits for ethanol production. Society for Industrial Microbiology, Annual Meeting (New Orleans, July 24-28).

PART 5: Research Activities of FY-12 (January 1, 2012 to March 31, 2012).

Written by: Benedict Okeke and Ananda Nanjundaswamy.

Introduction

The objectives of this study were 1) optimization of enzyme production in lab-scale 'farm-deployable' bioreactor, 2) comparison of saccharification of switchgrass biomass by SG2 aqueous enzyme extract and commercial enzymes, 3) characterization of oligosaccharides in saccharified switchgrass biomass by LC-MS, and 4) molecular size exclusion chromatography of cellulolytic and xylanolytic enzymes produced by SG-2 fermentation by fast protein liquid chromatography (FPLC), to estimate molecular size of enzymes.

Previously, a 20L capacity lab-scale 'farm-deployable' bioreactor was designed and developed for enzyme production and saccharification. The process was further optimized for production of SG2 enzymes. *Trichoderma* SG2 aqueous enzyme (crude) extract and commercially used enzymes for saccharification of biomass were compared in this study to short list a treatment for further evaluation in lab-scale bioreactor.

Materials and methods:

Enzyme production in laboratory model low-cost bioreactor

Design of low-cost farm-deployable bioreactor

A borosilicate carboy (herein referred to as bioreactor) 20 L capacity was custom fitted with VWR Symphony high-capacity stirrers, PID controlled temperature controlling system, custom-made silicone heating blanket wrapped around the bioreactor and temperature controlled by a digi-sense controller (Cole-Palmer), PT 100 temperature probe and an air pump (Tetra Whisper Air Pump).

Assembling and Operation of Bioreactor

About 10 L of optimized production medium (6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, 1000ml water and 2ml of mineral elements solution Focht (1994), was placed in the bioreactor and two silicone tubes (5 mm ID) were carefully immersed into the fermentation broth. One silicone tube was used as a sampling port while the other was fitted with a 0.45μm air filter. A 2" magnet was immersed in the medium to spin the liquid during fermentation. A PT100 temperature probe (Cole Parmer) was also immersed in the fermentation medium before autoclaving. Carefully all the tubes and temperature probe were bundled in a cotton plug and the opening of the bioreactor was closed using the cotton plug. The cotton plug was wrapped with an aluminum foil to prevent wetting during autoclaving. Tube ends were closed using a rubber band to prevent any spillage of medium during autoclaving. The bioreactor was carefully lowered into the vertical autoclave and sterilized for 60 min at 121°C. Upon cooling the bioreactor was carefully placed on a high capacity stirrer and stirring speed was set to 600 rpm. Air pump was connected to the air filter and air was pumped into the medium. The heating blanket was wrapped around the bioreactor and temperature probe connected to the controller where the temperature was set to 30°C. Once the set temperature was reached, approximately 1% of 72h grown SG-2 seed inoculum was added quickly into the bioreactor and the fermentation was carried out at 30°C for 7 days. Approximately 10 ml of samples were drawn from two bioreactors every 24h and were used for determining the enzyme activities.

Seed inoculum Preparation

About a loop full of SG-2 culture was inoculated into 50ml sterile high-solids medium in a 250ml flask containing 5% all-purpose flour (Pillsbury), 0.1% yeast extract and 0.1% peptone. Flasks were incubated at 30°C for 72h at 200rpm.

Enzyme activities
Enzyme activities were determined using previously described (see F11 second quarter report).

Saccharification
Approximately 100 g of acid-pretreated switchgrass was added to the broth and the temperature was increased to 50°C. About 10ml broth was collected every 24h until 72h for quantification of sugars released upon saccharification. During saccharification about 20mg of lactrol (Virginiamycin) was added to prevent bacterial growth.

Fermentation for ethanol production

Inoculum preparation
About one liter of *Sacchromyces cerevesiae* inoculum was grown in sterile medium containing 0.5 % glucose, 0.1% each of peptone and yeast extract for 72 hr. Inoculum was allowed stand for two hours prior to inoculation for cells to sediment by gravity. The supernatant was discarded and the cells with remaining liquid was collected and centrifuged. The supernatant was discarded and cells were re-suspended in sterile water and inoculated into the bioreactor.

Fermentation
The stirring speed was lowered to 300rpm to create a partial anaerobic condition that would stimulate ethanol production. About 10ml of sample was collected every 24h until 72h and analyzed for ethanol production and sugar utilization.

Comparison of switchgrass saccharification and fermentation by crude and commercial enzymes:

Pretreatments

Acid Pretreatment
Acid pretreatment was carried out using 2% sulfuric acid. About 100g of switchgrass was soaked in 2% sulfuric acid and autoclaved at 121° C for 1h. After autoclaving the contents were allowed to cool to room temperature, the liquid drained using a cheese cloth and the acid-pretreated switchgrass washed several times with water to completely remove the acid residues. After 4-5 washes the pH of the liquid was tested and was washed further if the pH was less than 5. The washed switchgrass was dried at room temperature for 72h and stored in airtight ziplock bags.

Alkali pretreatment
Alkali pretreatment was carried out using 2% sodium hydroxide. About 100g of switchgrass was soaked in 2% sodium hydroxide solution and autoclaved at 121° C for 1h. After autoclaving the contents were allowed to cool to room temperature, the liquid drained using a cheese cloth and the alkali-pretreated switchgrass washed several times to completely remove the alkali residues. After 4-5 washes pH of the liquid was tested and it was washed even further if the pH was higher than 7. The washed switchgrass was dried in room temperature for 72h and stored in airtight ziplock bags.

Seed inoculum Preparation
About a loop full of SG-2 culture was inoculated into 50ml sterile high solids medium in a 250ml flask containing 5% all-purpose flour, 0.1% yeast extract and 0.1% peptone. Flasks were incubated at 30°C for 72 at 200 rpm.

SG2 aqueous enzyme extract (crude) production
Crude enzymes were produced in ten 250ml flasks using optimized screening medium. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH2PO4, 1.2g (NH4)2SO4, 0.5g MgSO4.7H2O, 0.1g CaCl2, 0.003g FeSO4.7H2O, and 2ml of Fotch mineral element solution). Each flask contained 50ml medium and

flasks were sterilized at 121°C for 1h before inoculation. About 10 % of 72h SG-2 inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5th day the broth was transferred into sterile 50 ml centrifuge tubes and spun at 5000rpm for 10 min and the supernatant was pooled from 10 tubes, and approximately 500ml of crude enzyme extract was used for saccharification study. Enzyme samples were assayed for cellulose, xylanase, beta-glucosidase and beta-xylosidase.

Saccharification

Saccharification was carried out in 100ml flask with airtight caps. About 10ml of reaction mixture was taken in each flask. About 2% of switchgrass (virgin, autoclaved, acid-pretreated or alkali-pretreated) was added in each flask. All experiments were carried out in duplicates. Novozyme enzymes cellulase (NS22086), xylanase (NS22083), β-glucosidase (NS22118) and Hemicellulase (NS22002) were used as commercial enzyme source. Enzyme dosing was adjusted as per the recommendation by Novozymes (cellulase 5%, xylanase 0.25%, β-glucosidase 0.6% and hemicellulose 2%) for 2-5% (w/v) biomass suspension.

Enzyme treatments included (i) **C** crude aqueous extract of SG-2, (ii) **N** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase), (iii) **C50N** SG-2 crude extract and 50% Novozyme recommended dosage and (iv) **C25N** SG-2 crude extract and 25% Novozyme recommended dosage. Saccharification was carried out at 50°C at 80rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All the flasks were closed tightly to avoid escape of moisture. About 0.5ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Ethanol fermentation

After 72h, 100µl of 48h old *Saccharomyces cerevisiae* inoculum was added and the flasks were incubated at 30°C on the shaker at 65rpm. At 24h interval up to 72 samples was drawn from flaks to quantify for ethanol production.

All the samples after sugar and ethanol quantification were preserved for further characterization of oligosaccharides using LCMS.

LCMS characterization of Oligosaccharides

Shimadzu LCMS 2020 equipped with dual probe ionization system (DUIS) with two high pressure LC 20AD pump, SIL-20A autosampler, CBM-20A, SPD-20A detector and CTO 20A column oven was employed for the oligosaccharide characterization. Method described by Liu et al (2005) was employed with minor modifications.

Mobile phase consisting of 65% of acetonitrile in 0.1% formic acid was used with a flow rate of 0.2 ml/min. Nitrogen was used a nebulizing gas as well as drying gas at a flow rate of 13l/min. The heating block temperature and DL temperature were maintained at 400°C and 250°C respectively. For ionization ESI technique was employed with positive polarity detection. Approximately 2 µl of the sample was injected in and scanned for oligosaccharides between ranging 300-950 m/z value for 2 min. Standards (0.1mg/ml) of four oligosaccharides namely cellobiose, cellotriose, cellotetraose and cellopentaose were procured from Sigma Aldrich.

Size-exclusion chromatography of cellulolytic and xylanolytic enzymes by fast protein liquid chromatography (FPLC)

Sample preparation

Crude extract: SG2 enzymes were produced by growing on screening medium for 7 days and broth was harvested and centrifuged at 5000 rpm. Supernatant was used for enzyme purification as crude extract.

Acetone precipitation
Cold enzyme extract was added to cold acetone at 50: 50 ratio and the precipitated proteins were centrifuged and reconstituted using acetate buffer.

Molecular Sieving/ Gel filtration
For purification of enzymes Biorad BioLogic Dual flow FPLC system was used. Gel filtration chromatography using GE Superdex TM 200 10/300 GL was employed. Elution of proteins was carried out using buffer (50 mM potassium phosphate, 0.15M NaCl, buffer pH 7.0). About 2ml of samples were collected. Based on the UV spectrum, sample tubes (1-15) were selected and assayed for enzyme activities (cellulase, carboxymethyl cellulase, xylanase, beta-glucosidase and beta-xylosidase). Standard proteins with known molecular weights were used as reference.

Results

Enzyme production in laboratory model low-cost bioreactor
Large-scale fermentor for crude enzyme production (Fig. 1) yielded highest activities (U/ml/30min) of 1.72, 70.33, 0.25 and 1.05 for cellulase, xylanase, β-glucosidase and β-xylosidase respectively (Fig. 2a-c) on days 5, 6, 4 and 6 respectively. Enzyme activities were, however, much higher in flasks (Table 1) than observed in preliminary studies using the lab model bioreactor.

Comparison of switchgrass saccharification and fermentation by crude and commercial enzymes
The activities of SG2 crude and commercial (Novozyme) enzymes are listed in Table 1.

Overall, the statistical analysis (SAS 9.2) indicated that pretreatment of switchgrass significantly influenced glucose and xylose release ($P<0.0001$) from biomass. To further dissect the influence of switchgrass pretreatment, ANOVA of each pretreatment was carried out separately for glucose and xylose using by statement in PROC GLM (Table 2).

Virgin switchgrass
Novozyme enzymes resulted in highest glucose release (Fig. 3a) which was significantly greater than all other enzyme treatments. Sugar release from C, C25N and C50N were not significantly different from each other. The maximum glucose yield of 1.97±0.03 g/L was achieved at 72h saccharification of untreated (virgin) switchgrass using commercial Novozymes cocktail. Xylose production also followed a similar trend (Fig. 3b), with maximum yield of 0.79±0.01 g/L after 72h of saccharifcation with Novozymes cocktail.

Autoclaved switchgrass
Novozyme enzymes resulted in highest glucose release (Fig. 4a) and all enzyme treatments were significantly different from each other (N>C50N>C25N>C). The maximum glucose yield of 2.51±0.05 g/L was achieved at 72h saccharification of autoclaved switchgrass using commercial Novozymes cocktail followed by 2.18±0.14 g/L by C50N. Novozymes enzyme cocktail resulted in highest xylose release (Fig. 4b) which was significantly greater than that from SG2 crude enzyme but was not different from either C25N or C50N. Maximum yield of 1.0g/L xylose was released from N, C25N and C50N.

Alkali-pretreated switchgrass
Glucose release was not affected by enzyme treatments, but the highest glucose release of 9.0±0.68 g/L was released from C50N (Fig. 5a). The lack of statistical significance implies that there exists large variation and the experiment needs to be validated. The highest xylose release of 3.3g/L was from C25N and C50N (Fig. 5b). There was significant variation of the data for the alkali pre-treated biomass. The high sugar release and large variation suggests that the experiment with the alkali treated biomass needs to be validated.

Acid-pretreated switchgrass

C50N resulted in highest glucose yield (Fig. 6a) and all enzyme treatments were significantly different from each other (C50N>C25N>N>C). The highest yield was 5.61±0.1 g/L by C50N after 48h saccharification and remained unchanged after 72h. The next best yield of 5.11±0.3g/L was due to C25N after 72h. A similar trend was seen for xylose release (Fig. 6b) and the highest xylose yield of 1.64±0.02 g/L was seen after 72h saccharification by C50N.

Ethanol fermentation

Ethanol production was seen only in all enzyme treatments of acid-pretreated switchgrass (Fig. 7). The highest ethanol yield of 2.12±0.06 g/L was seen after 24h fermentation of acid-pretreated switchgrass sample. Only two enzyme treatments (N and C50N) resulted in negligible ethanol production of 0.61 and 0.09 g/L respectively.

LCMS characterization of oligosaccharides

Molecular spectra of the standard oligosaccharides namely cellobiose, cellotriose, cellotetraose and cellopentaose used in this study are outlined (Fig. 8a-d) and their molecular weights are 365.3, 527.44, 689.58 and 851.72 Da respectively. Preliminary screening of the acid-pretreated samples saccharified with four different enzymes (C, N, C50N and C25N) at 24h (Fig 9a-d) and 72h (Fig 10a-d) shows that all the four short listed oligosaccharides are present in the samples.

Size-exclusion chromatography of cellulolytic and xylanolytic enzymes by fast protein liquid chromatography (FPLC)

Activity profiles of SG2 cellulolytic and xylanolytic enzymes namely cellulase, xylanase, CM-cellulase, beta-glucosidase and beta-xylosidase in fractions from 1-15 are presented in Fig 11a and b. The elution profiles of standard proteins and the SG2 acetone precipitation sample are outlined in Figs 12a and b respectively. Calibration of the column with gel filtration protein standard is presented in Fig 13.

Table 1. Activities of SG2 and commercial enzymes

Enzyme	Activity (U/ml/30min)
SG2 Cellulase	11.3
Novozyme cellulase	31.46
SG2 xylanase	108.61
Novozyme xylanase	55.21
SG2 beta glucosidase	16.75
Novozyme beta glucosidase	24.72
SG2 beta xylosidase	13.45

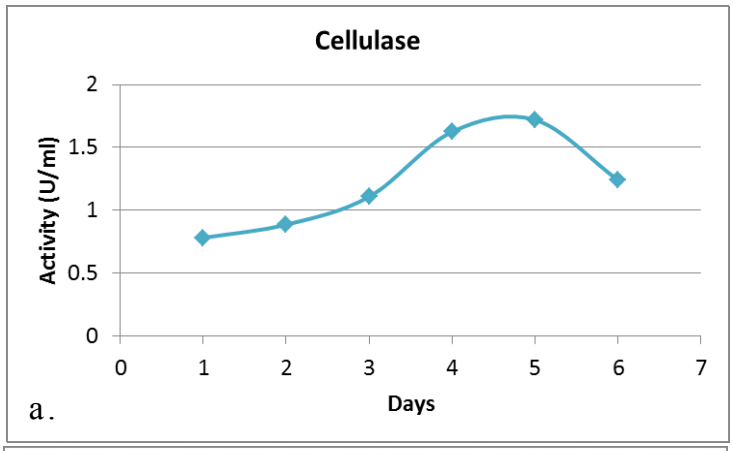
Table 2. ANOVA for the effect of pretreatment and enzyme on saccharification of switchgrass

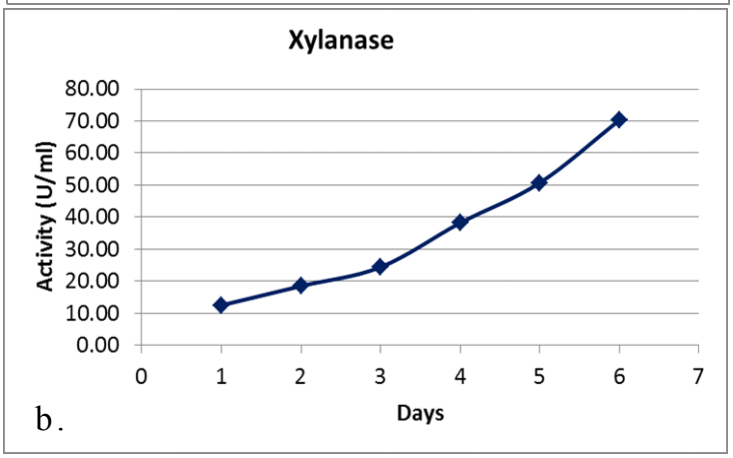
Glucose release			Xylose release		
Pretreatment	Factor Enzyme		Pretreatment	Factor Enzyme	
	F	**P**		**F**	**P**
Virgin	46.84	<0.0001	Virgin	11.67	<0.0001
Autoclaved	90.96	<0.0001	Autoclaved	56.45	<0.0001
Alkali	2.11	0.1363	Alkali	54.52	<0.0001
Acid	60.24	<0.0001	Acid	19.00	<0.0001

Figure 1

Figure 1. . Submerged fermentation for crude enzyme production in 20-L laboratory model bioreactor.

Figure 2





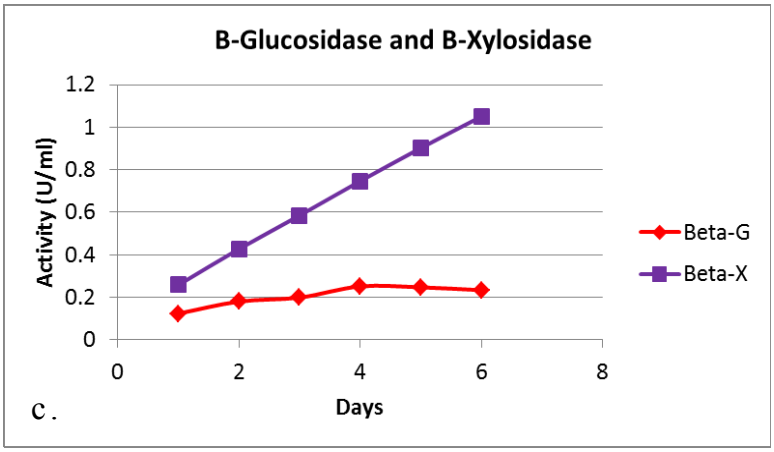


Figure 2a-c. SG-2 crude enzyme production in 20L laboratory model.

Figure 3

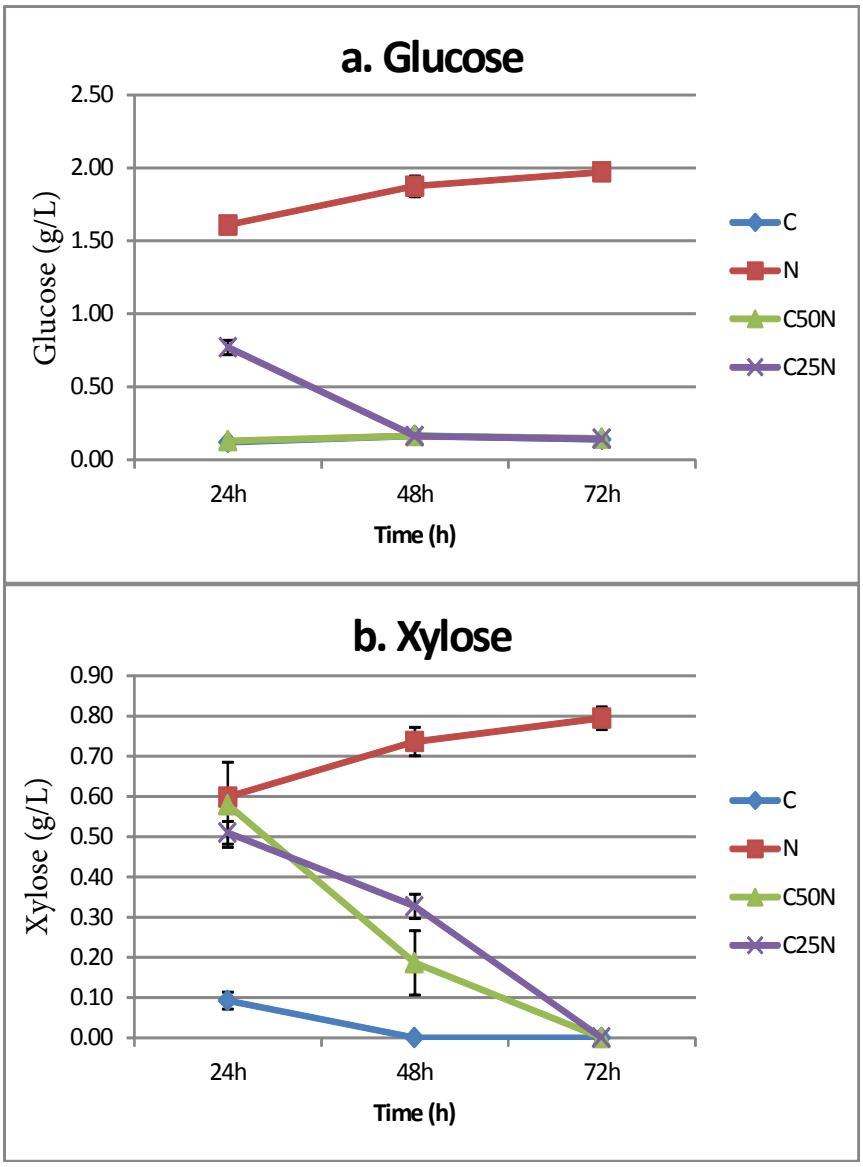


Figure 3. Saccharification of virgin switchgrass. a. glucose production b. xylose production. Means and standard errors are provided. ***C*** crude aqueous extract of SG-2; ***N*** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase) ; ***C50N*** SG-2 crude extract and 50% Novozyme recommended dosage and ***C25N*** SG-2 crude extract and 25% Novozyme recommended dosage.

Figure 4

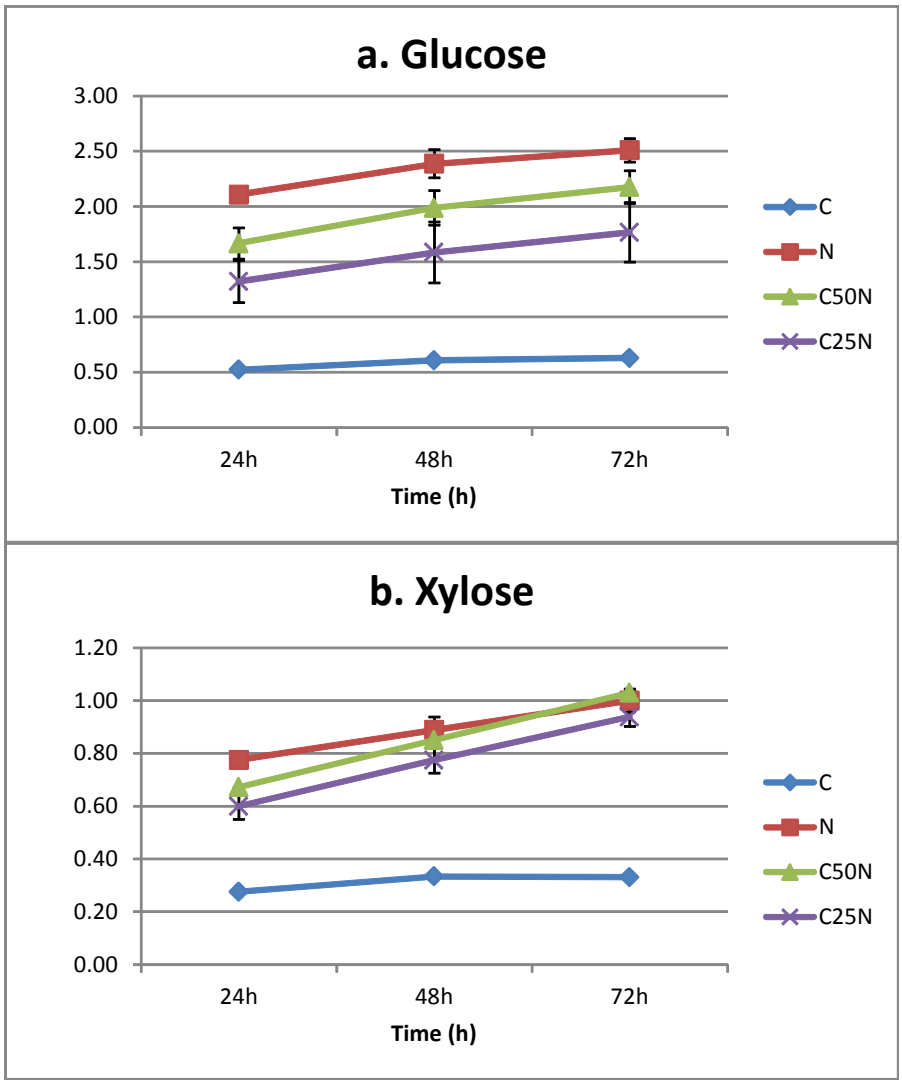


Figure 4. Saccharification of autoclaved switchgrass. a. glucose production b. xylose production. Means and standard errors are provided. ***C*** crude aqueous extract of SG-2; ***N*** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase) ; ***C50N*** SG-2 crude extract and 50% Novozyme recommended dosage and ***C25N*** SG-2 crude extract and 25% Novozyme recommended dosage.

Figure 5

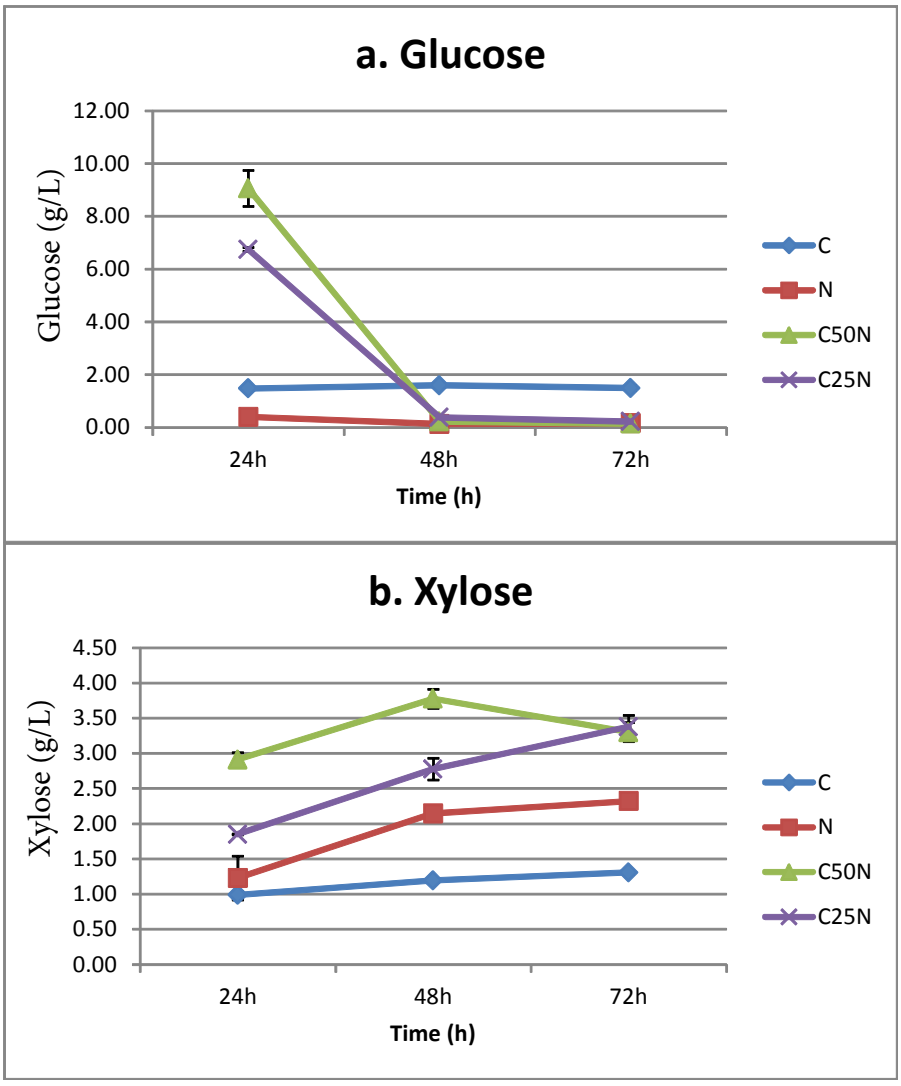


Figure 5. Saccharification of alkali-pretreated switchgrass. a. glucose production b. xylose production. Means and standard errors are provided. ***C*** crude aqueous extract of SG-2; ***N*** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase) ; ***C50N*** SG-2 crude extract and 50% Novozyme recommended dosage and ***C25N*** SG-2 crude extract and 25% Novozyme recommended dosage.

Figure 6

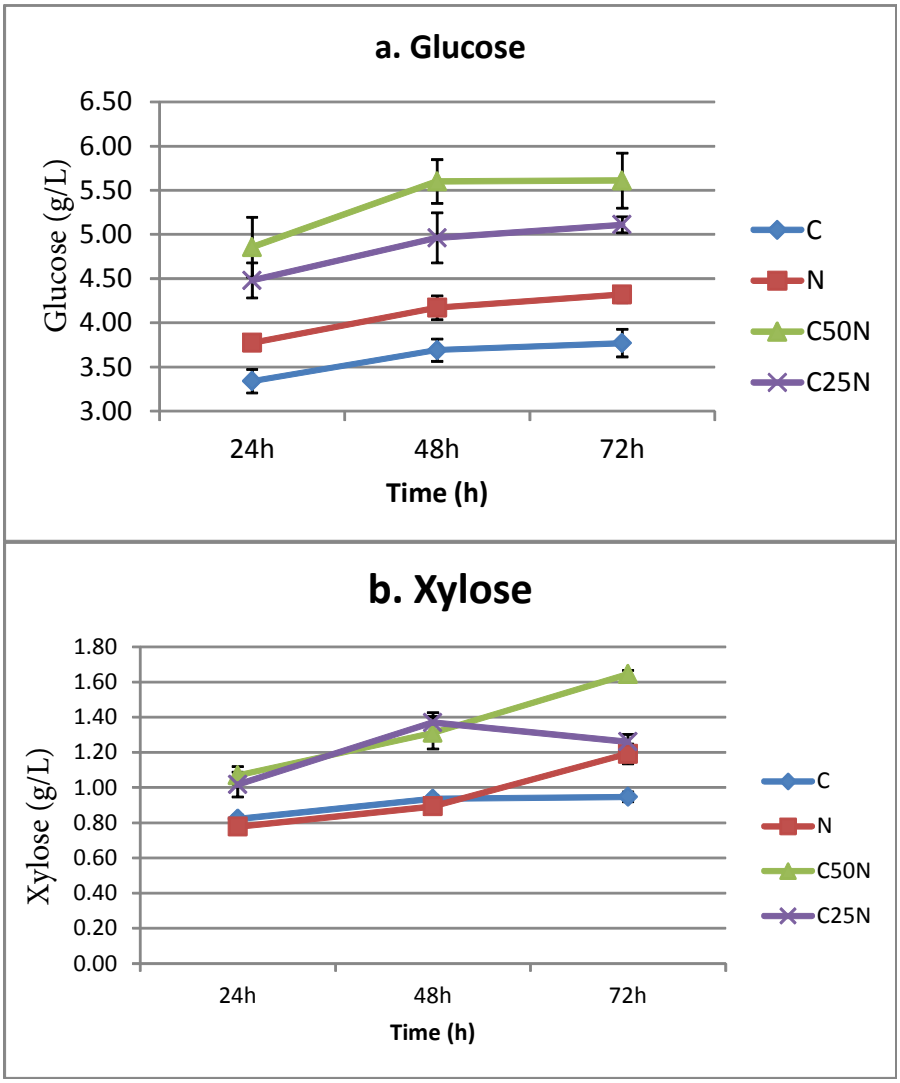


Figure 6. Saccharification of acid-pretreated switchgrass. a. glucose production b. xylose production. Means and standard errors are provided. ***C*** crude aqueous extract of SG-2; ***N*** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase) ; ***C50N*** SG-2 crude extract and 50% Novozyme recommended dosage and ***C25N*** SG-2 crude extract and 25% Novozyme recommended dosage.

Figure 7

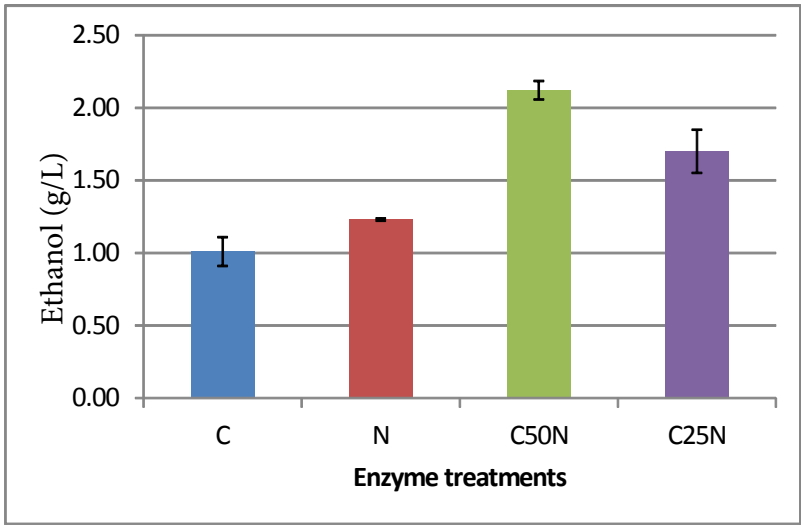


Figure 7. Ethanol production from sugars produced from acid-pretreated switchgrass. Means and standard errors are provided. ***C*** crude aqueous extract of SG-2; ***N*** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase) ; ***C50N*** SG-2 crude extract and 50% Novozyme recommended dosage and ***C25N*** SG-2 crude extract and 25% Novozyme recommended dosage.

Figure 8a, b

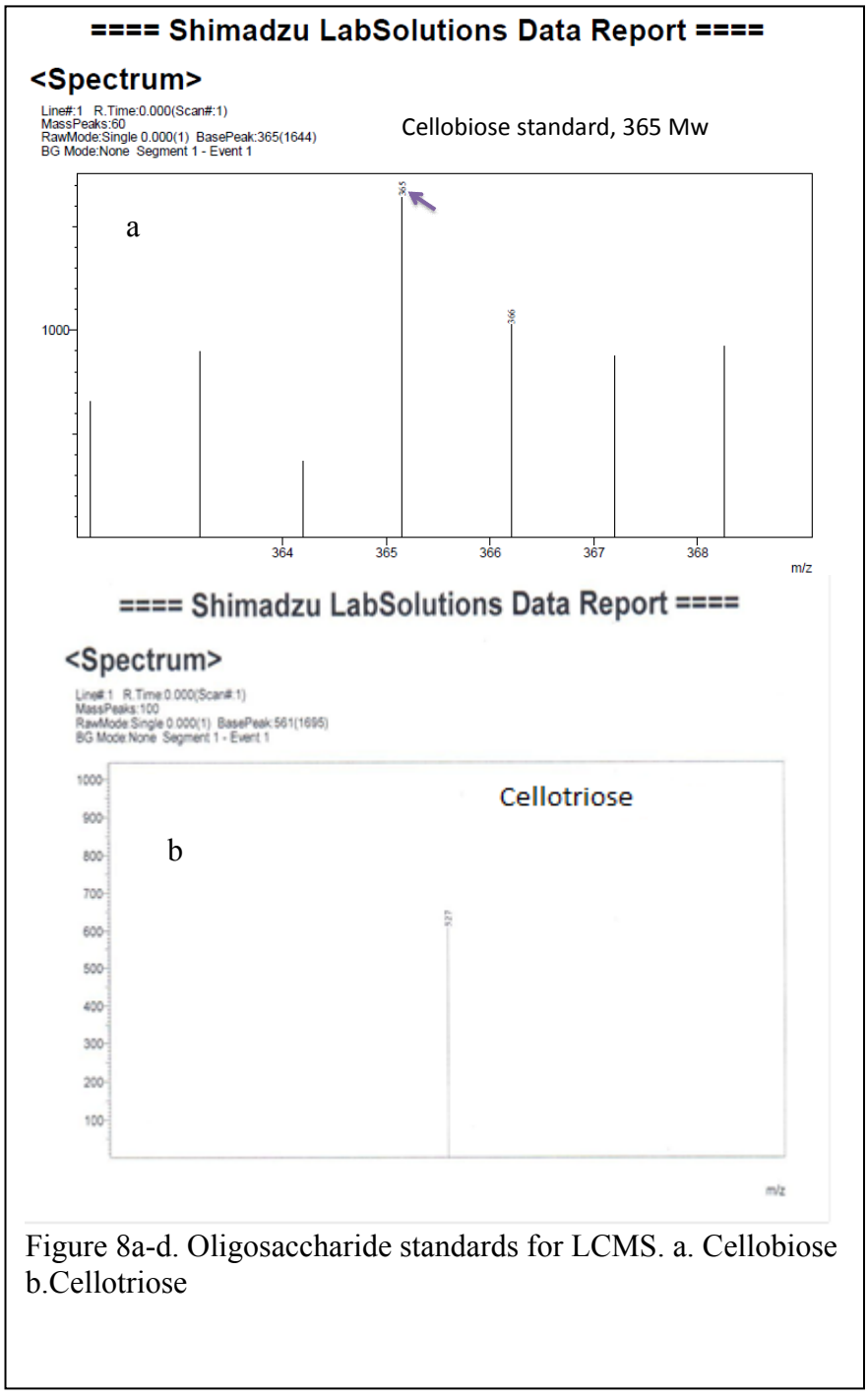


Figure 8a-d. Oligosaccharide standards for LCMS. a. Cellobiose b.Cellotriose

Figure 8c, d

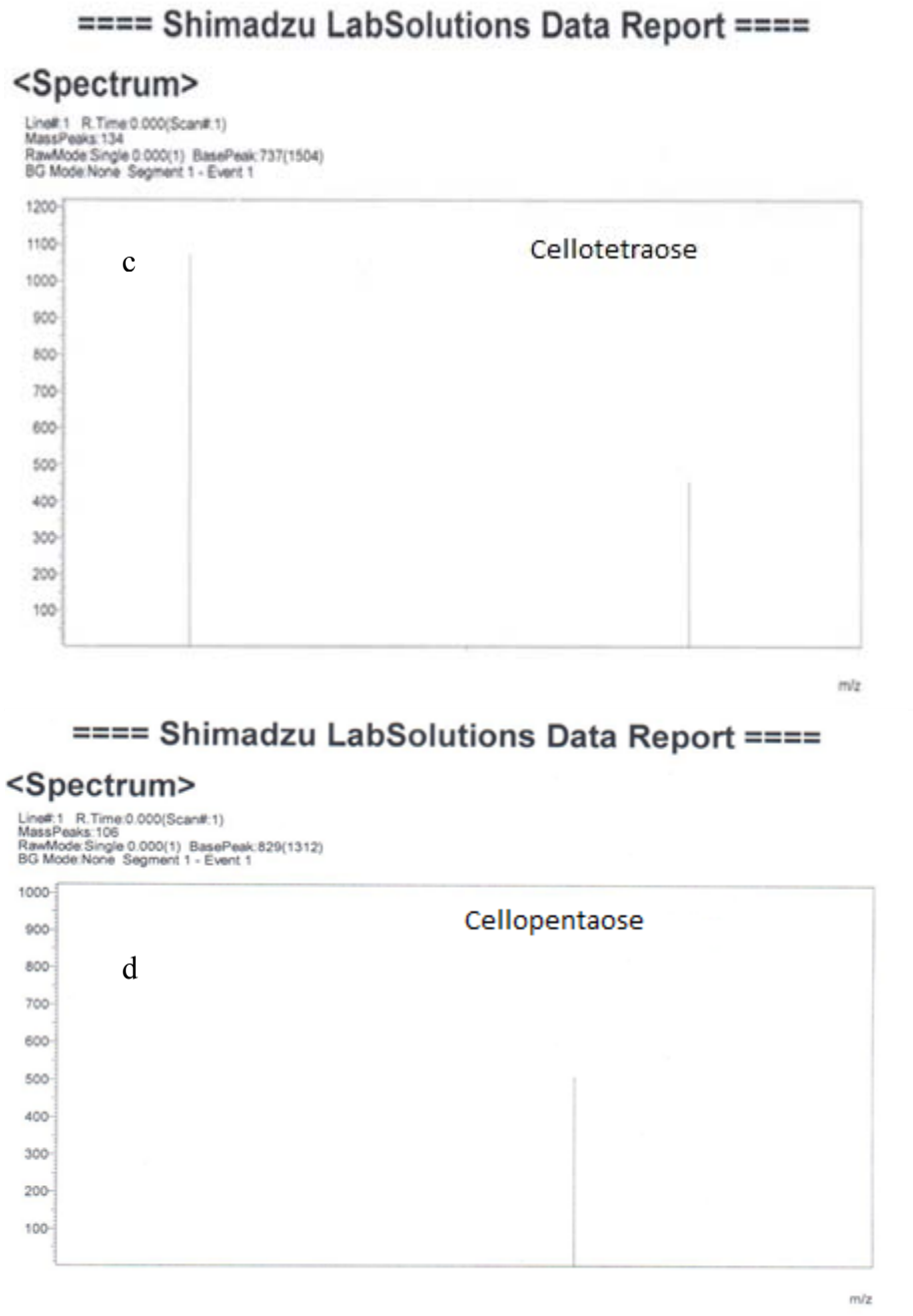


Figure 8. c. Cellotetraose standard Mw= 689.44 d. Cellopentaose standard Mw=851.72

Figure 9a, b

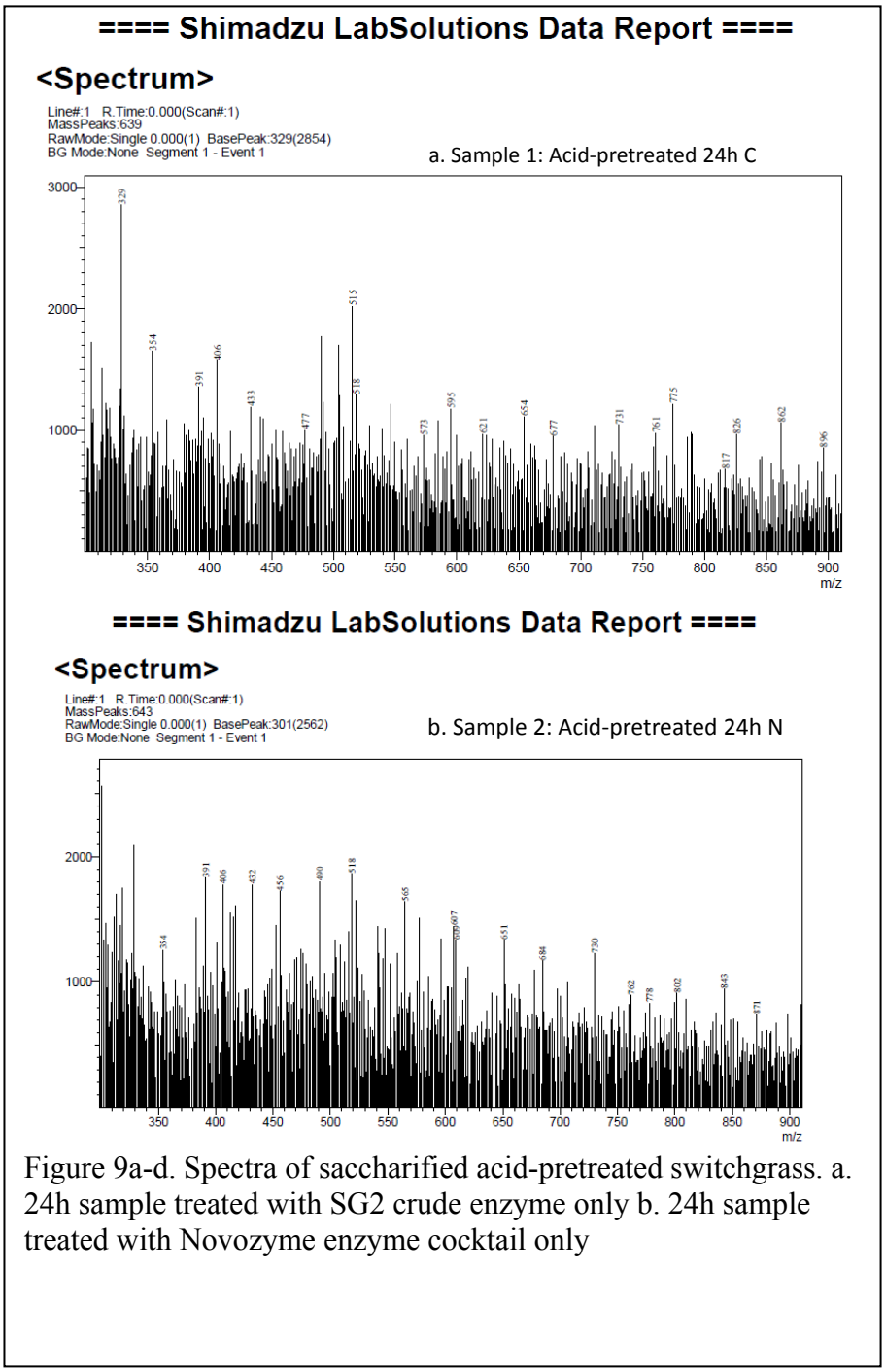


Figure 9a-d. Spectra of saccharified acid-pretreated switchgrass. a. 24h sample treated with SG2 crude enzyme only b. 24h sample treated with Novozyme enzyme cocktail only

Figure 9c, d

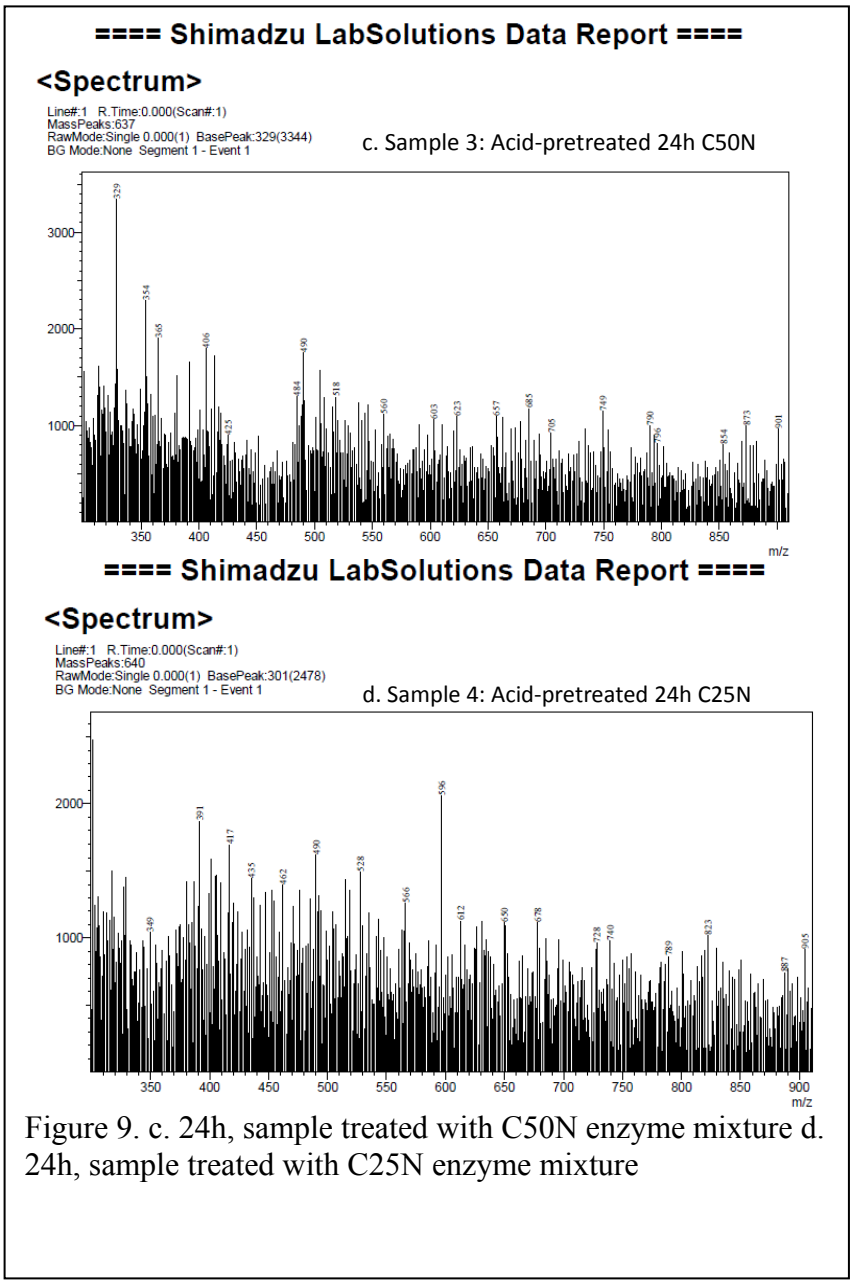


Figure 9. c. 24h, sample treated with C50N enzyme mixture d. 24h, sample treated with C25N enzyme mixture

Figure 10a, b

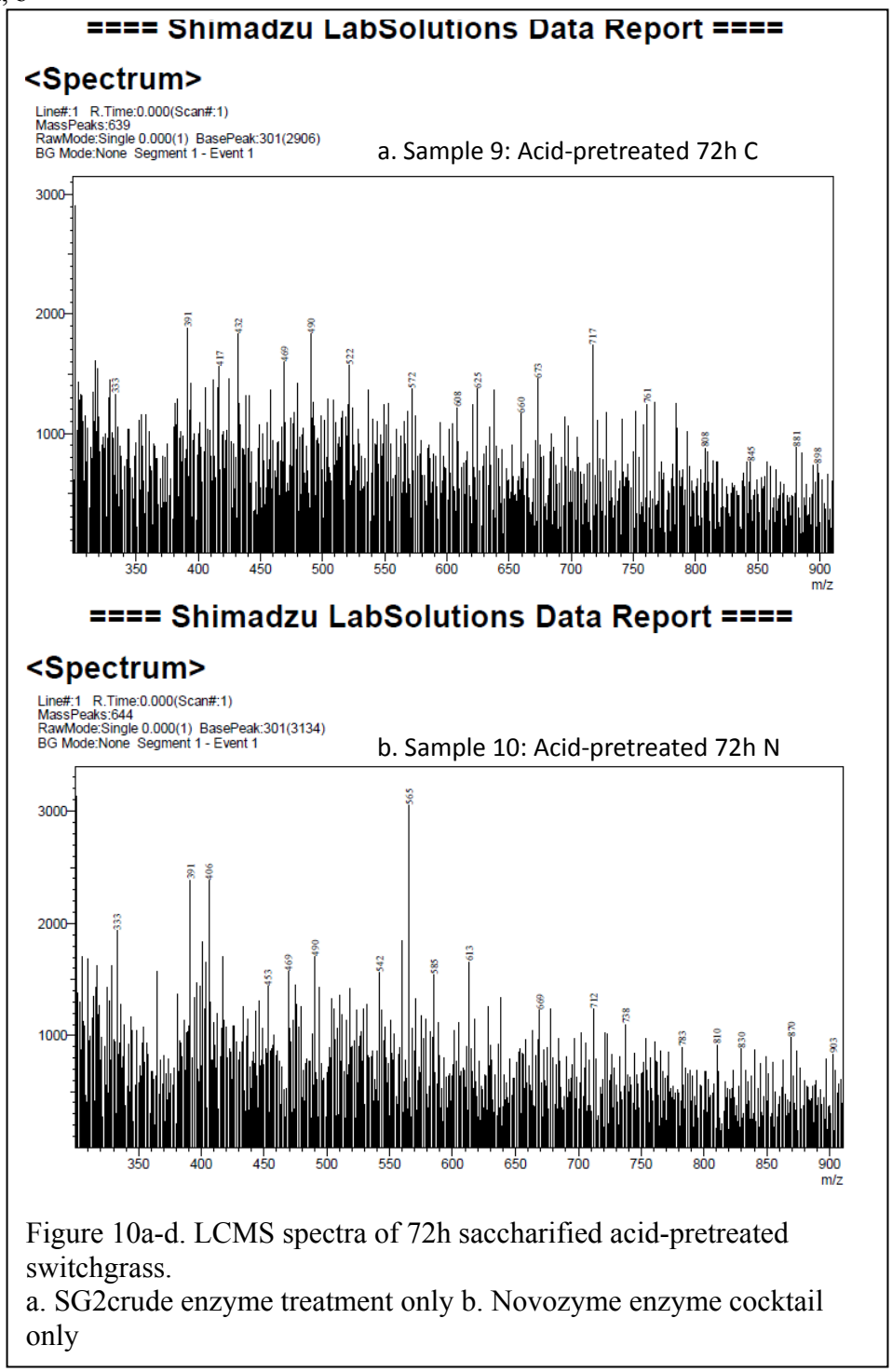


Figure 10a-d. LCMS spectra of 72h saccharified acid-pretreated switchgrass.
a. SG2crude enzyme treatment only b. Novozyme enzyme cocktail only

Figure 10c, d

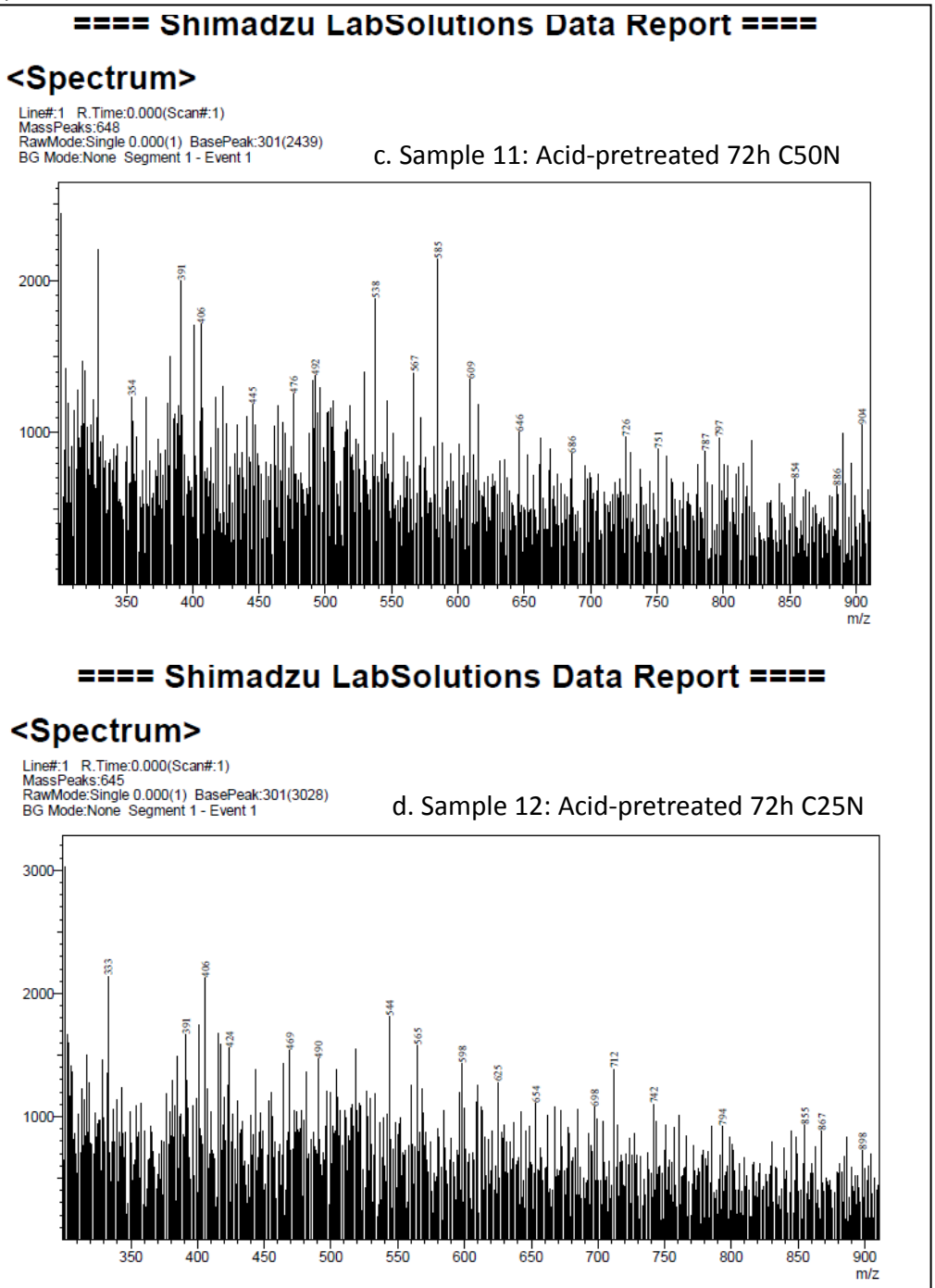


Figure 10. c. C50N enzyme mixture treated sample d. C25N enzyme mixture treated sample

Figure 11

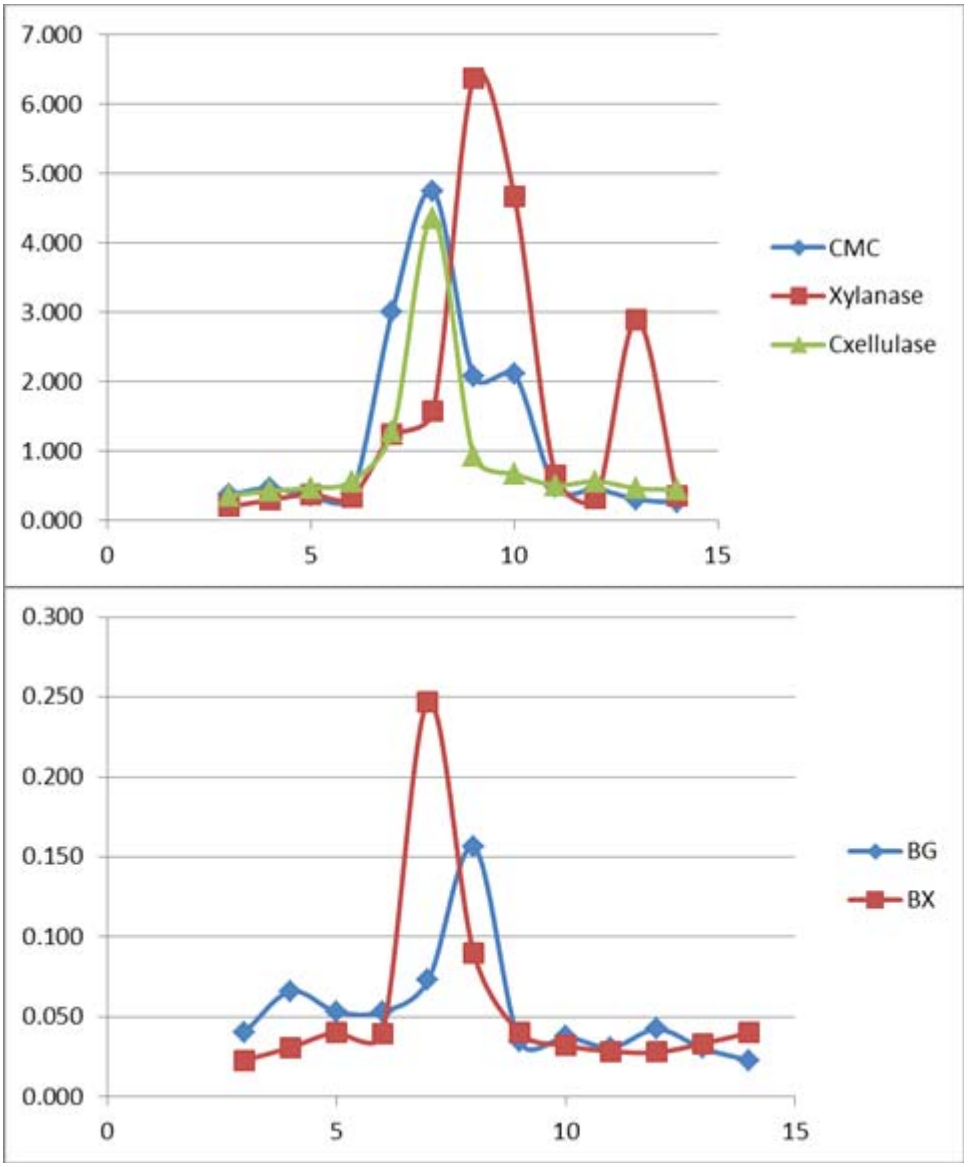


Figure 11. Enzyme fractions from SG2 extract. a. Cellulase, xylanase and Carboxymethyl cellulase activities. X-axis=enzyme fractions, Y-axis = enzyme activities (Units/mL)

Figure 12

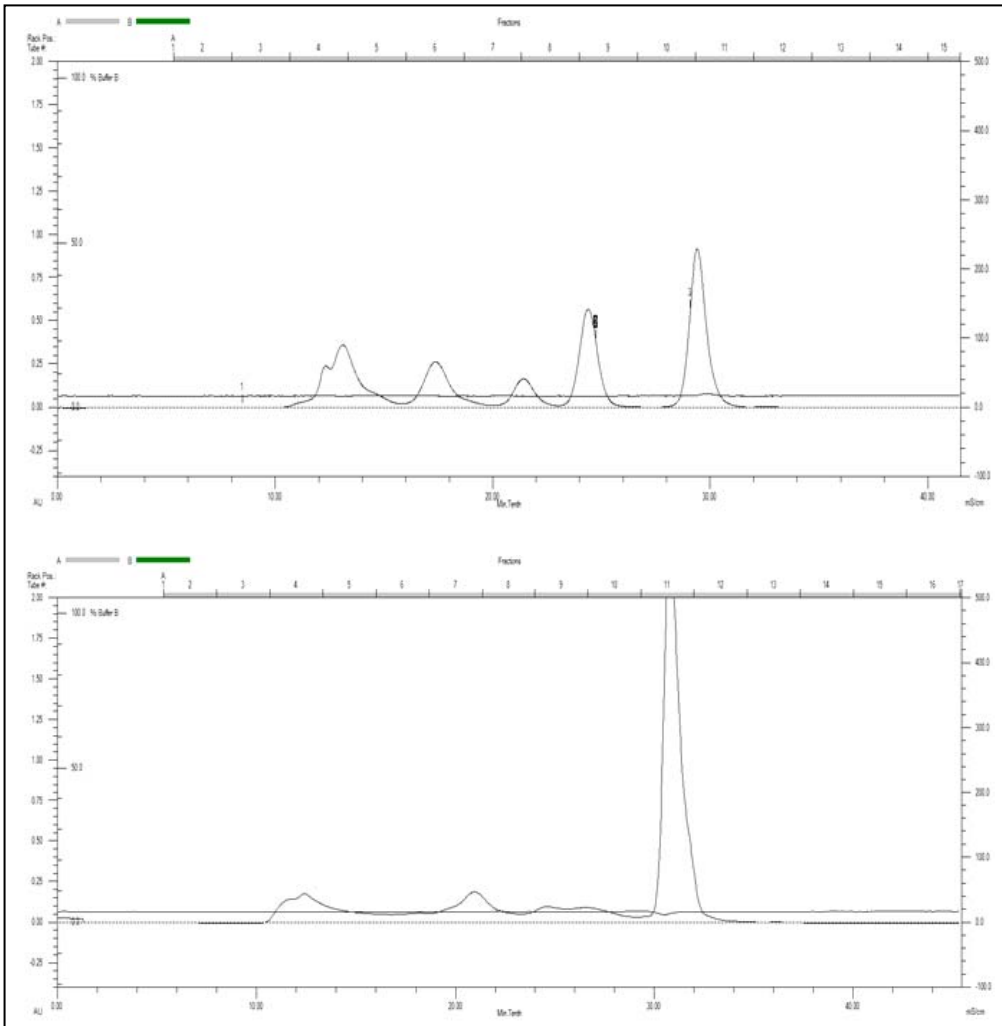


Figure 12. FPLC profiles. a. Standard proteins b. Acetone precipitated SG2 enzyme sample

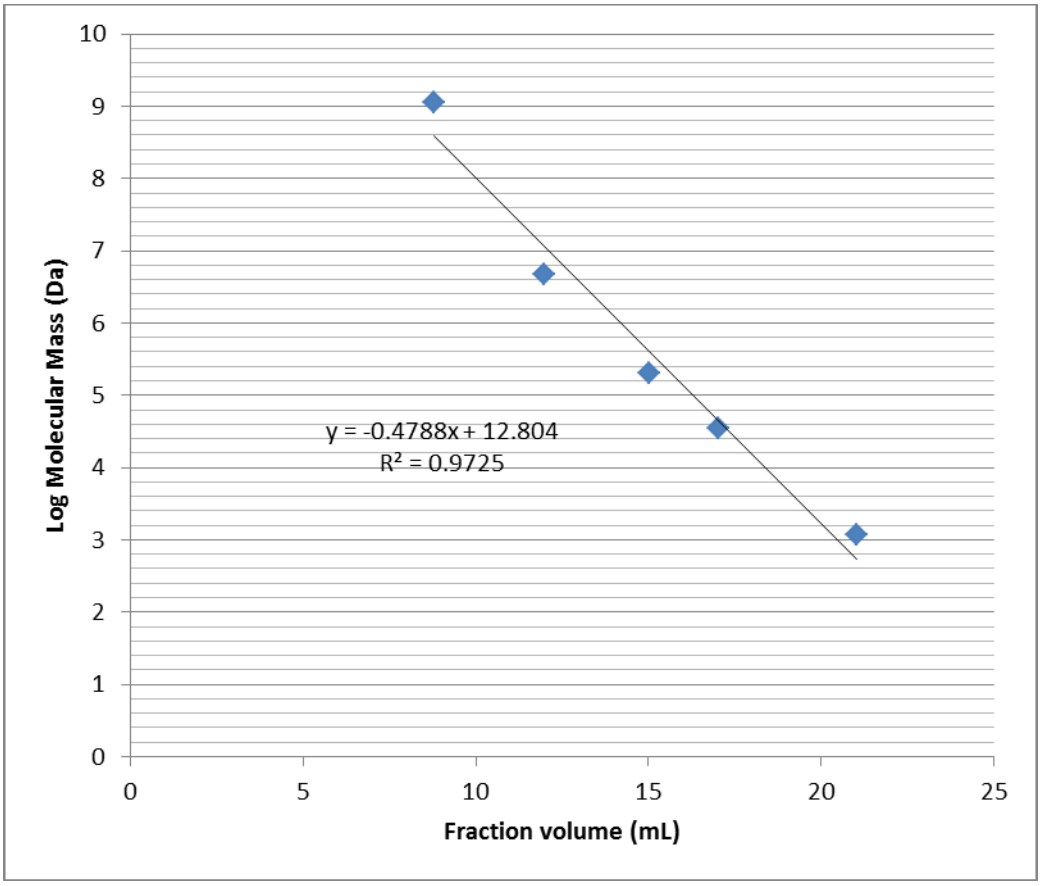


Figure 13: Estimation of molecular mass of cellulolytic and xylanolytic enzymes by size exclusion chromatography (gel filtration). Five protein standards on the graph arranged from the highest to the lowest size (Da) are Thyroglobulin (670,000), γ-globulin (158,000), Ovalbumin (44,000), Myoglobin (17,000) and Vitamin B12 (1,350)

Conclusions and Milestones

Enzyme production in laboratory model low-cost bioreactor

- The activities of SG2 cellulase, xylanase, beta glucosidase and beta xylosidase in shake flasks (table 2) were 6.6 times, 1.5 times, 67 times and 12.8 times greater than that in lab-scale bioreactor respectively. The low activities in large-scale bioreactor are most likely due to the limited mixing and aeration.

Comparison of switchgrass saccharification and fermentation by crude and commercial enzymes

- Novozyme cellulase and beta glucosidase had higher activities than that of SG2 crude enzymes whereas SG2 xylanase had twice the activity of that from Novozymes.
- Overall, acid pretreated switchgrass upon saccharification resulted in highest sugar yield followed by autoclaved switchgrass and then by virgin switchgrass. Results from alkali pretreated switchgrass need to be validated due to large variation in some data points.
- Further, in the acid pretreated switchgrass, C50N after 72h saccharification resulted in highest sugar yield. The average cellulose content of switchgrass is in the range of 32-40% (NREL 2004). A combination of *Trichoderma* SG2 crude enzyme supplemented with Novozyme enzymes at 25% or 50% of recommended dosage (C25N or C50N respectively) resulted in 19% to 30% increase in glucose yield than that from commercial enzyme alone.

- Supplementation of commercial enzyme with SG2 crude enzyme at 50% recommended dosage can potentially make the process cost-effective (using only half the recommended dosage of commercial enzymes) and improve process efficiency (at least 30% more glucose yield).
- Since SG2 crude enzyme is only an aqueous extract of the fermentation broth, no expensive chemicals or processes are employed ensuring an inexpensive production of crude enzyme.
- Maximum fermentation efficiency of 74% was seen in C50N, followed by 65% with C25N, 55% by N commercial enzyme only and 52% with C crude enzyme alone, respectively.

LCMS characterization of oligosaccharides

- The four short listed oligosaccharides were present in the samples after 24, 48 and 72h of saccharification with different enzymes.

Size-exclusion chromatography of cellulolytic and xylanolytic enzymes by fast protein liquid chromatography (FPLC)

- From the activity profiles, it was clear that the cellulolytic and xylanolytic enzyme activities were in fractions 5-11 (Fig 11 and 12). A possible residual xylanase activity was detected around fractions 12-13. The fraction numbers with the peak activity for most enzymes were about those of Myoglobin (17,000 kDa) and greater but less than the size of γ-globulin (158,000 kDa). This suggests that membranes with molecular mass cut off of ≤ 10 kDa can be employed for concentration of the enzymes. Bakare et al (2005) reported that cellulases from *Pseudomonas fluorescence* had molecular weight of 26 and 36kDa. Wipusaree et al (2011) reported that xylanase of a fungal isolate *Alternaria alternata* displayed a molecular size of 54.8kDa. Karnchanatat et al (2007) reported a molecular size of 64.2kDa for the beta-glucosidase of *Daldinia eschscholzii*.

Presentations:

1. Nanjundaswamy, A. and Okeke, B. (2012). *'All-in-one' bioprocessing strategy for cellulosic ethanol production: a laboratory model bioreactor study*. Auburn University Research Week, Auburn, AL, April 2-4, 2012.
2. Okeke, B., Prescott, A., Deravi, Y., Bishop, J., Peaks, S., Sawyer, L., Nanjundaswamy, A. and Hall, R. (2012). Screening of xylose-utilizing yeasts and bacteria for xylose fermentation to ethanol. Auburn University Research Week, Auburn, AL, April 2-4, 2012.
3. Deravi Y, Prescott A, Peaks S, Sawyer L, Bishop J, Nanjundaswamy, A, Hall, R. and (2012). Production dynamics of cellulolytic-xylanolytic enzymes complex of *Fusarium oxysporum* FS22A and *Trichoderma amazonicum* FS5A. Auburn University at Montgomery (AUM) Undergraduate Research, Symposium.

Specific Milestones

Sub-Task A.1: Milestone (A.1.ML.1): Complete selection of fungi. Selected fungi are expected to produce 25 g of sugar from 200 g of substrate per liter. The following analysis indicate that this milestone has been achieved with acid pre-treated biomass. Data obtained with untreated (virgin) biomass is also encouraging. Further studies are in progress.

- About 0.75g of glucose was saccharified from 20g of virgin switchgrass at 24h of incubation with crude enzyme and 25% of Novozyme enzyme dose. This will result in 7.5g of sugar from 200g of switchgrass.

- About 0.5 g of xylose was saccharified from 20g of virgin switchgrass at 24h of incubation with crude enzyme and 25% of Novozyme enzyme dose. This will result in 5.0g of sugar from 200g of switchgrass.
- From 200g of virgin switchgrass there is a potential to obtain a total of **12.5g** of total sugar consisting of both glucose and xylose**.**
- Assuming that virgin switchgrass contains 40% cellulose (NREL, 2004), 200g of the material will contain 80g of cellulose and this will result in 7.5g of glucose.
- A combination of crude and 50% of Novozyme enzyme resulted in **5.61g** of glucose from **20g** of acid pretreated switchgrass at 48h. This indicates a potential yield of 56.10g of glucose from **200g** of acid pretreated biomass.
- A combination of crude enzyme extract and 50% Novozyme enzyme will result in 1.64g of xylose from 20g of acid pretreated biomass. This will indicate that a potential yield of 16.4g of xylose from 200g of acid pretreated switchgrass.
- Acid pretreated switchgrass has the potential to result in a total of **72.5g** of total sugars consisting of glucose and xylose from 200g.
- Autoclaved switchgrass when treated with combination of crude and 50% of Novozyme enzyme resulted in 2.18g of glucose from 20g of switchgrass. This indicates a potential yield of 21.8g of glucose from 200g of switchgrass.
- Autoclaved switchgrass when treated with combination of crude and 25 % or 50% of Novozyme enzyme resulted in 1.00g of xylose from 20g of switchgrass. This indicates a potential yield of 10.0g of xylose from 200g of switchgrass.
- There is a potential to obtain about **31.8g** of total sugar (glucose and xylose) from 200g of autoclaved switchgrass.
- Generally A.1.ML.1 has been achieved in flask but requires scale up and optimization in laboratory scale bioreactor.

References:

1. Ananda N, Vadlani PV, Vara Prasad PV (2011). Drought and heat stressed grain sorghum (*Sorghum bicolor*) does not affect the glucose and ethanol production. Industrial Crops and Products 33:779-782
2. Bakare M.K. Adewale I. O., Ajayi A. and Shonukan O. O., (2005) Purification and characterization of cellulase from the wild-type and two improved mutants of Pseudomonas fluorescens Afr. J. Biotechnol. 4: 898-904
3. Focht D. D. (1994) In R. W.Weaver et al. (Eds.), Methods of Soil Analysis, Part 2 – Microbiological and Biochemical Properties. Soil Science Society of America, Book Series 5 (pp. 407-426). Madison, SSSA.
4. Karnchanatat A., Petsom A., Sangvanich P., Piaphukiew J., Whalley A.J., Reynolds C. D., Sihanonth P., (2007) Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm. FEMS Microbiol Lett. 270(1):162-70.

5. Liu Y, Urgaonkar S, Verkade J G, Armstrong D W. (2005) Separation and characterization of underivatized oligosaccharides using liquid chromatography and liquid chromatography-electrospray ionization mass spectrometry. J. of Chromatogr A 24;1079(1-2):146-52.

6. NREL (2004) Biomass Feedstock Composition and Properties Database, 2004, http://www.nrel.gov/biomass/energyanalysis.html

7. Wipusaree N., Sihanonth P., Piapukiew, J., Sangvanich P. and Karnchanatat, A. (2011) Purification and characterization of a xylanase from the endophytic fungus Alternaria alternata isolated from the Thai medicinal plant, Croton oblongifolius Roxb. Afr. J. Microbiol. Res. 5: 5697-5712.

PART 6: Research Activities of FY-12 (April 1, 2012 to June 30, 2012).
Written by: Benedict Okeke and Ananda Nanjundaswamy

Introduction:
In the last quarter among the different pretreatments used for switchgrass, acid-pretreated switchgrass upon saccharification resulted in highest sugar yield. Furthermore, a combination of *Trichoderma* SG2 crude enzyme supplemented with Novozyme (N) enzymes at 25% or 50% of recommended dosage (C25N or C50N respectively) resulted in 19% to 30% increase in glucose yield than that from commercial enzyme alone. Maximum ethanol conversion efficiency of 74% was seen in C50N, followed by 65% with C25N, 55% by N commercial enzyme only and 52% with C crude enzyme alone, respectively. Accordingly, in this quarter, two additional acid-pretreated lignocellulosic biomass-gammagrass and sawdust were saccharified using SG2 crude enzyme-commercial enzyme mixtures. Additionally, another cellulolytic and xylanolytic enzyme producing *Fusarium* FS22A from our collection was also employed for saccharification of acid-pretreated switchgrass, gammagrass and sawdust.

Materials Methods:
Effect of media volume on production of biomass-saccharifying-enzymes by *Trichoderma* SG2:
Crude enzyme extract was produced in nine 250 ml flasks using optimized screening medium. (One liter medium: 6.2 g powdered waste paper, 9.6 g pulverized switch grass, 1.4 g peptone, 0.6 g yeast extract, 0.5 g Tween 80, 2 g KH_2PO_4, 1.2 g $(NH_4)2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$, 0.003 g $FeSO_4.7H_2O$, and 2 ml of Fotch mineral element solution). The medium was optimized from that employed in a previous study (Okeke, 2012) using Fotch's mineral elements supplement (Fotch, 1994). Different volumes (50, 75 and 100 ml) of the screening medium for enzyme production by SG2 were evaluated. Triplicates of each treatment were employed. Flasks were sterilized at 121°C for 1 h before inoculation and about 2 % of the seed inoculum was used to inoculate the flasks. Flasks were incubated for 5 days, samples from flasks were centrifuged at 5000 rpm for 10 min and supernatant was used for testing enzyme activities.

Stability of *Trichoderma* SG2 enzyme activity
Fresh potato dextrose agar (PDA) plates were streaked with *Trichoderma* SG2, incubated at 30°C for one week for sporulation and used for inoculation seed medium. Seed inoculum was incubated at 30°C for 3d and was used to inoculate screening medium. Three 250 ml flasks containing 50 ml of the screening medium were incubated at 30 °C, 5 d and 200 rpm. Samples were centrifuged at 5000 rpm and supernatant was collected for determination of enzyme activities. This was repeated after 3 months to compare enzyme activities and determine the stability of *Trichoderma* SG2 culture.

Cellulolytic and xylanolytic enzyme production and saccharification by *Trichoderma* SG4, *Trichoderma* FS5A and *Fusarium* FS22A:
Seed inoculum preparation: About a loop full of the respective culture was inoculated into 50 ml sterile high solids medium in a 250 ml flask containing 5% all-purpose flour, 0.1% yeast extract and 0.1% peptone. Flasks were incubated at 30°C for 72 h at 200 rpm.

Enzyme production medium:
Crude enzyme production was carried out in 250 ml flasks containing optimized screening medium. (One liter medium: 6.2 g powdered waste paper, 9.6 g pulverized switch grass, 1.4 g peptone, 0.6 g yeast extract, 0.5 g Tween 80, 2 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$, 0.003 g $FeSO_4.7H_2O$, and 2 ml of Fotch mineral element solution). Each flask contained 50 ml medium and flasks sterilized at 121°C for 1h before inoculation. About 10% of 72h inoculum was added into each flask aseptically, incubated at 30°C for 5 days. The crude enzyme samples obtained on days 2, 4 and 5 were assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase activities. On 5th day of enzyme production, the broth was transferred into sterile 50 ml centrifuge tubes and centrifuged at 5000 rpm for 10 min. The supernatant was used for biomass saccharification.

Saccharification
Saccharification was carried out in 100ml flasks with airtight caps. About 10 ml of crude enzyme extract was placed in each flask. About 2% of acid-pretreated switchgrass was added to each flask. All experiments were carried out in duplicates. Saccharification was carried out at 50°C at 80 rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All the flasks were closed tightly to avoid escape of moisture. About 0.5 ml of sample was drawn at 24 h interval up to 72 h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Saccharification of biomass by crude enzymes of *Trichoderma* SG2 and *Fusarium* FS22A
Pretreatment
Acid pretreatment of switchgrass, gammagrass and sawdust was carried out using 2% sulfuric acid. About 100 g of the respective biomass was soaked in 1L of 2% sulfuric acid and autoclaved at 121°C for 1h. After autoclaving the contents were allowed to cool to room temperature, liquid drained using a cheese cloth and the acid-pretreated switchgrass washed several times with water to completely remove the acid residues. After 4-5 washes the pH of the liquid was tested and was washed further if the pH was less than 5. The washed biomass was dried at room temperature for 72h and stored in airtight ziplock bags.
Moisture Determination:
Moisture determination of virgin and pretreated biomass was carried out using Mettler Toledo Moisture meter (HB 43-5 Halogen). About 1 g of the sample (pretreated and control) was placed in the aluminum pan and moisture content was recorded in % by following the preloaded method in the instrument. At least two readings were recorded for each of the samples.

SG2 and FS22A aqueous enzyme extract (crude) production
Crude enzymes were produced in six 250 ml flasks using optimized screening medium. (One liter medium: 6.2 g powdered waste paper, 9.6 g pulverized switch grass, 1.4 g peptone, 0.6 g yeast extract, 0.5 g Tween 80, 2 g KH_2PO4, 1.2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$, 0.003 g $FeSO_4.7H_2O$, and 2 ml of Fotch mineral element solution). Each flask contained 50 ml medium and flasks were sterilized at 121°C for 1h before inoculation. About 2% of 72h SG-2 or FS22A inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5th day the broth was transferred into sterile 50 mL centrifuge tubes and centrifuged at 5000 rpm for 10 min. Supernatant from respective fungi was pooled and the crude enzyme extract was used for saccharification. The crude enzyme samples were assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase.

Saccharification
Saccharification was carried out in 100 ml flasks with airtight caps. About 10 ml of reaction mixture was taken in each flask. About 2% of the respective acid-pretreated biomass was added to each flask. All experiments were carried out in duplicates. Novozyme enzymes cellulase (NS22086), xylanase (NS22083), β-glucosidase (NS22118) and Hemicellulase (NS22002) were used as commercial enzyme source. Enzyme dosing was adjusted as per the recommendation by Novozymes (cellulase 5 units per %TS, xylanase 0.25, β-glucosidase 0.6 and hemicellulase 2 respectively).

Enzyme treatments included (i) **C** crude aqueous extract of SG-2 or FS22A, (ii) **C25N** SG-2 or FS22A crude extract and 25% Novozyme recommended dosage (iii) **C50N** SG-2 or FS22A crude extract and 50% Novozyme recommended dosage (iv) **N** Novozyme enzyme recommended dosage (v) **N25** 25% of Novozyme recommended dosage and (vi) **N50** Novozyme at 50% recommended dosage. Novozyme enzymes recommended dosage included 5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase. Saccharification was carried out at 50°C at 80rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All the flasks were closed tightly to prevent loss of moisture. About 0.5 ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Ethanol fermentation
After 72h, 100µl of 48h old *Saccharomyces cerevisiae* inoculum which was washed in sterile water and resuspended in sterile water was added and the 100 ml flasks containing about 10ml saccharified sample were incubated at 30°C on a shaker at 65 rpm. Samples were drawn after 24h, 48h and 72h to quantify ethanol production.

Scale-up switchgrass saccharification and ethanol fermentation

Enzyme production
Crude enzyme extract of *Trichoderma* SG2 was produced in ten 250ml flasks using optimized screening medium. (One liter medium: 6.2 g powdered waste paper, 9.6 g pulverized switch grass, 1.4 g peptone, 0.6 g yeast extract, 0.5 g Tween 80, 2 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution). Each flask contained 100 ml medium and flasks were sterilized at 121°C for 1h before inoculation. About 2% of 72h inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5^{th} day the broth was transferred into sterile 250 ml centrifuge tubes, centrifuged at 4000 rpm for 20 min, supernatant pooled from all tubes, and approximately 1000 ml of crude enzyme extract was used for saccharification study. The crude enzyme samples were assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase.

Saccharification
Saccharification was carried out at 50°C in a 5 L glass media bottle with airtight cap. About 1L of the SG2 crude enzyme extract was transferred to the bottle to which 20 g of acid-pretreated switchgrass was added. Novozyme enzymes cellulase (NS22086), xylanase (NS22083), β-glucosidase (NS22118) and Hemicellulase (NS22002) were used as commercial enzyme source. Enzyme dosing was adjusted to 50% of the recommended dosage (cellulase 5 units per %TS, xylanase 0.25, β-glucosidase 0.6 and hemicellulase 2

respectively). Periodically samples were collected at 24, 48 and 72h and analyzed for sugar release.

Ethanol fermentation

After 72h, 100 µl of 48h old *Saccharomyces cerevisiae* inoculum which was washed in sterile water and resuspended in sterile water was added and the 5 L glass media bottle was incubated at 30°C on a shaker at 65 rpm. Samples were drawn at 24h, 48h and 72h to quantify sugar release and ethanol production. This experiment was repeated twice.

Statistical analyses

Statistical Analysis Software (SAS, version 9.3) was used for data analyses. When required, Analysis of Variance (ANOVA) was used to compare treatments. PROC GLM was used and pair-wise differences were ascertained using *Tukey* correction at $P=0.05$.

Results and Discussion

Effect of media volume on production of biomass-saccharifying-enzymes by *Trichoderma* SG2

None of the three volumes tested exerted any significant impact on enzyme production by SG2 (Fig. 1).

Stability of *Trichoderma* SG2 for production of enzymes

The cellulase, xylanase and beta xylosidase enzyme activities produced by strain SG2 did not decrease after three months (Fig. 2).

Cellulolytic and xylanolytic enzyme production and saccharification by *Trichoderma* SG4, *Fusarium* FS5A and *Fusarium* FS22A

a) **Enzyme activities:** The enzyme activities of SG4, FS5A and FS22A are outlined in Figs 3a-d. Enzyme activities increased with time for all three microbes except for beta xylosidase for FS5A which reduced by day 5. After 5d of enzyme production, cellulase activities in FS22A and SG4 were similar and that of FS5A the least (Fig. 3a), xylanase activities of all three microbes were similar (Fig. 3b), SG4 produced best beta glucosidase activity followed by FS5A and least in FS22A (Fig. 3c) and finally, SG4 produced the highest beta xylosidase activity (Fig. 3d).
b) **Saccharification of acid-pretreated switchgrass:** Overall, FS22A resulted in highest glucose release followed by SG4 and the least in FS5A ($F=122.38$, $P<0.0001$; $FS22A^{a}>SG4^{b}>FS5A^{c}$). Further analysis by each fungus showed that release of sugar increased over time for all three fungi with the maximum glucose released at 72h followed by 48h and least after 24h of saccharification ($72h^{a}>48h^{b}>24h^{c}$; $P<0.001$; Fig. 4a-b). The highest glucose yield of 3.5g/L and xylose yield of 1.22g/L after 72h saccharification of acid-pretreated switchgrass was recorded with FS22A.

Saccharification of biomass by crude enzymes of *Trichoderma* SG2 and *Fusarium* FS22A

a) **Enzyme activities of *Trichoderma* SG2 and *Fusarium* FS22A** The cellulase, xylanase, beta-glucosidase and beta-xylosidase activities of SG2 and FS22A were

recorded before the enzymes were used for saccharification of biomass (Fig. 5). Although the enzyme activities of both fungi were similar, SG2 recorded higher activities than that of FS22A. The activity of SG2 beta-xylosidase was five times higher than that of FS22A.

b) **Moisture determination:** The moisture content in virgin biomass varied from about 8.7% in switchgrass to 14.4% in sawdust with 7.6% in gammagrass (Fig. 6). Upon acid pretreatment, moisture content increased to 8.8% and 8.4% respectively in switchgrass and gammagrass, whereas it reduced to 10.9% in sawdust.

c) **Saccharification by FS22A:** Overall analysis of the saccharification by FS22A of three biomass tested showed that switchgrass yielded highest glucose followed by gammagrass and least in sawdust ($F=10.42$, $P=0.0001$). Further, in all three biomass the highest saccharification resulted from C50N and saccharification after 72h resulted in numerically the highest sugar yield but was statistically similar to that at 48h (Gammagrass: $P<0.0001$; $C50N^{a}>C^{b}>N^{c}>C25N^{c}$; $72h^{a}>48h^{a}>24h^{b}$; Sawdust: $P<0.0001$; $C50N^{a}>C25N^{b}>C^{c}>N^{d}$; $72h^{a}>48h^{a}>24h^{b}$; Switchgrass: $P<0.0001$; $C50N^{a}>C25N^{b}>N^{c}>C^{c}$; $72h^{a}>48h^{a}>24h^{b}$). Finally, the sugar release for each biomass within a time point shows that C50N was the best combination for maximum saccharification (Figs. 7-9).

d) **Saccharification by SG2:** Overall analysis of the saccharification by SG2 of three biomass tested showed that switchgrass yielded highest glucose followed by gammagrass and the least was sawdust ($F=84.94$, $P<0.0001$). Further, in all three biomass the highest saccharification resulted from C50N except in switchgrass where C25N and C50N was the highest (Gammagrass: $P<0.0001$; $C50N^{a}>C25N^{b}>C^{c}>N^{d}>N50^{e}>N25^{f}$; Sawdust: $P<0.0001$; $C50N^{a}>C25N^{b}>C^{c}>N^{d}>N50^{e}>N25^{f}$; Switchgrass: $P<0.0001$; $C25N^{a}>C50N^{a}>N^{b}>N50^{c}>C^{d}>N25^{e}$;) and saccharification after 72h resulted in the highest sugar yield in all three biomass and the least after 24h ($72h^{a}>48h^{b}>24h^{c}$). Finally, the sugar release for each biomass within a time point shows that C50N was the best combination for maximum saccharification except for switchgrass where C50N and C25N were the best (Figs. 10-12).

e) **Comparison of *Trichoderma* SG2 and *Fusarium* FS22A:** Overall, the sugar yield in sawdust ($F=6.17$, $P=0.0181$) and switchgrass ($F=4.04$, $P=0.0524$) by FS22A was significantly higher than that from SG2 whereas in gammagrass SG2 yielded higher glucose than FS22A ($F=58.92$, $P<0.0001$). As far as each enzyme treatment was concerned, in gammagrass: crude enzyme **C**, SG2>FS22A ($F=11.8$, $P=0.0063$); **C25N**, SG2>FS22A ($F=877.96$, $P=<0.0001$); **C50N**, SG2>FS22A ($F=299.12$, $P<0.0001$); saw dust: **C**, SG2=FS22A ($F=1.57$, $P=0.2389$); **C25N**, FS22A>SG2 ($F=17.21$, $P=0.002$), **C50N**, FS22A>SG2 ($F=80.33$, $P<0.0001$) and in switchgrass, **C**, FS22A>SG2 ($F=5.41$, $P=0.0424$), **C25N**, FS22A=SG2 ($F=0.01$, $P=.99$), **C50N**, FS22A>SG2 ($F=73.73$, $P<0.0001$).

f) **Comparison of Novozyme enzyme saccharification:** Overall, glucose yield from three biomass varied significantly upon using Novozyme enzymes and the trend was yield from switchgrass (SG) was significantly greater than that from gammagrass

(GG) which was greater than that from saw dust (SD) ($SG^a>GG^b>SD^c$ F=530.42 P<0.0001) and enzyme treatment also resulted in significantly different glucose yields across biomass, sugar yield by novozyme full strength yielded highest sugar compared to 50% enzyme which was significantly greater than that from 25% novozyme enzyme ($N^a>N50^b>N25^c$ F=109.43, P<0.0001). Expectedly, inclusion of 50% and 25% of Novozyme enzymes (N50 and N25) released 50% and 25% glucose respectively compared to that from recommended Novozyme dosage (N) in different substrates.

g) **Ethanol production in SG2 saccharified substrates:** Overall, the ethanol yield after 24h of yeast fermentation was statistically significant: the highest yield was from switchgrass followed by gammagrass and least in sawdust ($SG^a>GG^b>SD^c$, F=15.98, P<0.0001). Further analysis of effect of enzyme treatments on ethanol production within each substrate showed that in both switchgrass and gammagrass, the enzyme treatments had a significant effect and the highest ethanol was produced by Novozyme enzyme alone followed by 50% dilution of novozyme, followed by 25% dilution of novozyme and least in the crude enzyme (P<0.0001, $N^a>N50^b>N25^c>C50N^d>C25N^e>C^f$). In case of saw dust, no ethanol was produced in any of the enzyme treatments. Ethanol production was below detectable levels after 48h and 72h of biomass saccharification. Fig. 13a-c outlines the ethanol production along with residual sugar after 24h of fermentation. The highest ethanol production in switchgrass by SG2 was 0.73g/L with **N**, 0.28g/L with **N50**, 0.21g/L with **C50N**, 0.17g/L with **N25** and 0.14g/L with **C25N** with ethanol conversion efficiencies of 35%, 16.5%, 4%, 15% and 5% respectively. In gammagrass, the highest ethanol production was 0.42g/L with **N**, 0.2g/L with **N50** and 0.06g/L with **N25** respectively with ethanol conversion efficiencies of 44%, 26% and 15% respectively.

Scale-up switchgrass saccharification by C50N of SG2 and ethanol fermentation:

a) In scale-up saccharification of switchgrass using **C50N**, the glucose yield significantly varied over time: the highest yield was after 72h followed by 48h and least in 24h. The yield at 48h was not significantly different from that at 72h or 24h ($72h^a>48h^{ab}>24h^b$, P=0.0128). The maximum glucose yield of 4.97g/L and xylose yield of 1.37 g/L respectively were obtained from switchgrass after 72h saccharification (Fig. 14a).
b) After 24h ethanol fermentation, the ethanol yield was 2.0g/L and residual glucose was 0.13 g/L (Fig. 14b). Ethanol at 48h and 72h were below detectable levels. Residual glucose and xylose levels were 0.13 and 1.19g/L respectively.
c) Theoretical ethanol yield after 24h fermentation should have been 2.53g/L (4.97 g/L * 0.51). However, the actual yield was 2.0g/L which implies a conversion efficiency of about 80%.

Shaibani et al. (2011) reported that the crude enzymes produced by *Trichoderma longibrachiatum* and *Aspergillus niger* on solid state fermentation of bagasse successfully released 5.2g and 9.0g of glucose from 50g of bagasse respectively . In our study the best glucose yield of 5.26g was obtained from 20g of switchgrass with crude enzyme and 50% of the recommended dose of commercial enzyme (C50N). Wang et. al (2012) reported that crude enzyme extracts from their bacterial composite XDC-2 showed different saccharification profile in core corn stalk and rice straw. In general saccharification in rice straw was lower

than core of corn stalk. At 1% biomass loading rice straw yielded about 1g of soluble carbohydrates at 48h while core of corn stalk yielded about 2.1g of soluble carbohydrate at the same time. Ventila et.al. (2010) studied the saccharification profile of *Miscanthus* by three fungal strains, *Trichoderma viridae* (CMIT 3.5), *T. viridae* (CMGB1) and *A. niger* (Fluka 22178). Expectedly the degree of saccharification varied among them with *A. niger* (Fluka 22178) yielding total reducing sugar of 250mg/g of biomass and *T. viridae* (CMIT 3.5) resulted in the lowest sugar yield of about 20mg/g at 72h. They attributed the poor saccharification to the loss of enzyme activity at 4°C storage. Mukhopadhyay and Chatterje (2010) in their study with pre-treated water hyacinth obtained 18.28g of sugars from 40g of substrate during separate hydrolysis and fermentation. Ethanol yield was about 4.5g from 40g of water hyacinth biomass.

Presentations:

1. Nanjundaswamy A. and **Okeke B.C** (2012) 'All-in-one' bioprocessing strategy for cellulosic ethanol production: a laboratory model bioreactor study. International Biomass Conference and Expo, Denver, Colorado, April 16-19, 2012
2. **Okeke B.C.,** Nanjundaswamy, A., Deravi, Y., Peaks, S. Prescott, A. and Hall, R. (2012). Biomass saccharification by cellulolytic-xylanolytic enzymes complex of newly isolated fungal strains. SIM Annual Meeting and Exhibition, Washington DC, August 12-16, 2012 (Submitted).
3. Nanjundaswamy A., Hall R. and **Okeke B.C.** (2012): Lipid Production by Red Yeast Fermentation of Peanut Meal for Microbial Biodiesel (the study presented lipid accummulation by yeasts isolated from the project and potential esterification with lignocellulose ethanol to microbial biodiesel). American Society for Microbiology 2012 General Meeting, June 16-19, 2012, in San Francisco, California.

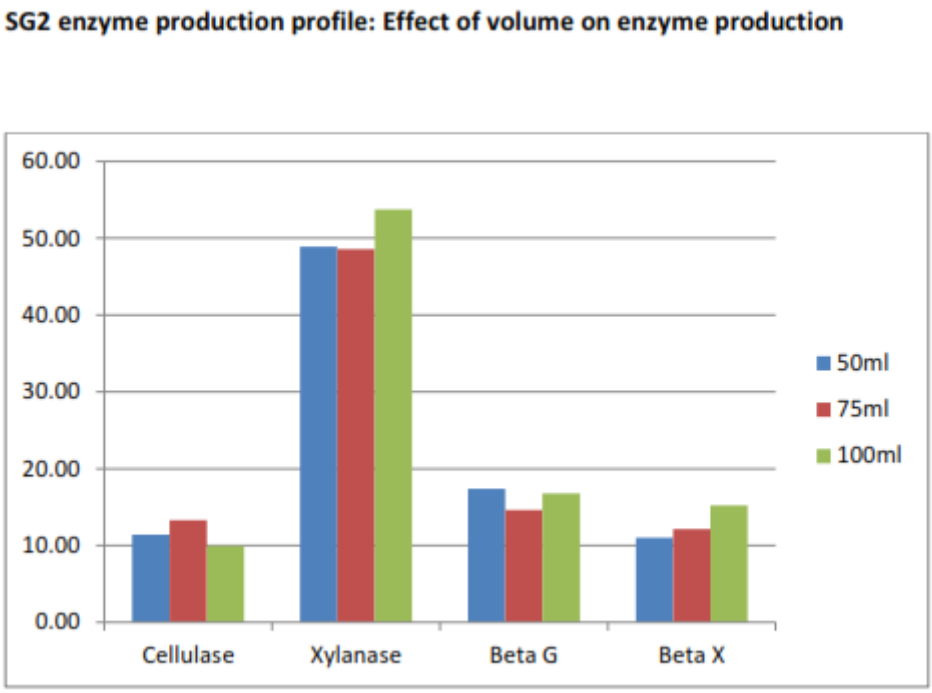


Figure 17 Effect of volume on enzyme production in Trichoderma SG2. X axis=Enzyme Y-axis= Media volume

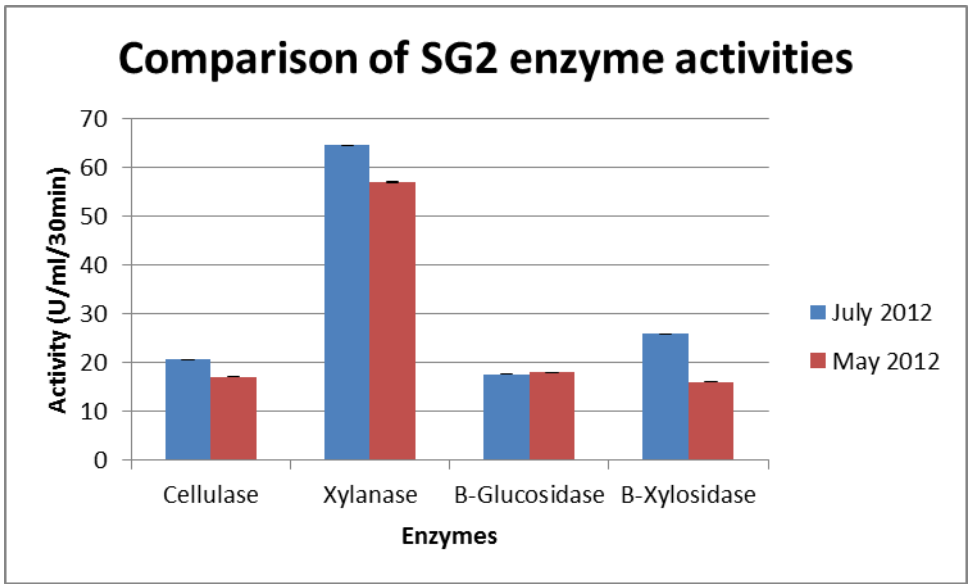
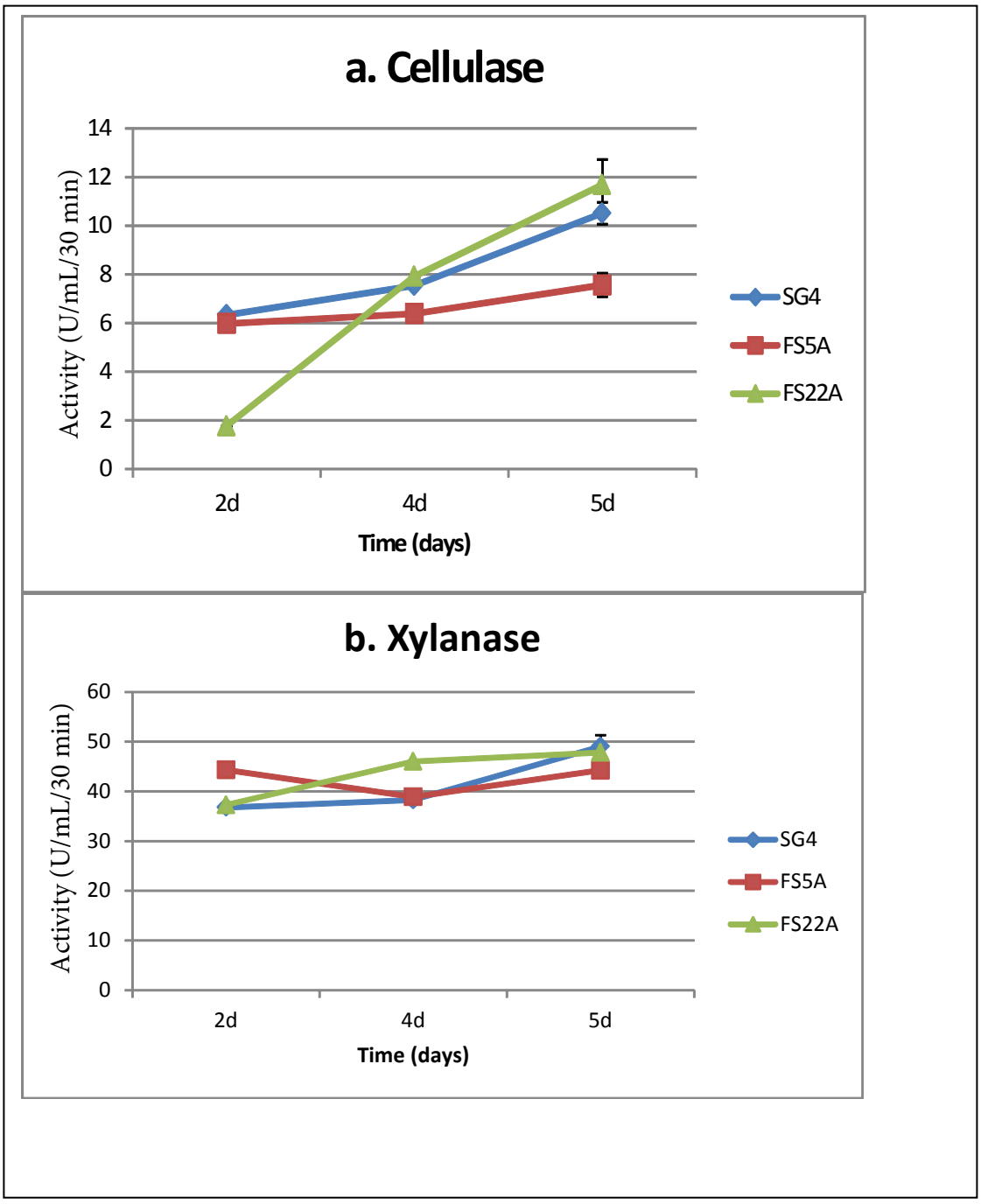


Figure 18 Stability of enzyme production by *Trichoderma* SG2. Mean activities and standard errors indicated.


a. Cellulase
Activity (U/mL/30 min)
14
12
10
8
6
4
2
0
2d
4d
5d
Time (days)
SG4
FS5A
FS22A
b. Xylanase
Activity (U/mL/30 min)
60
50
40
30
20
10
0
2d
4d
5d
Time (days)
SG4
FS5A
FS22A

Figure

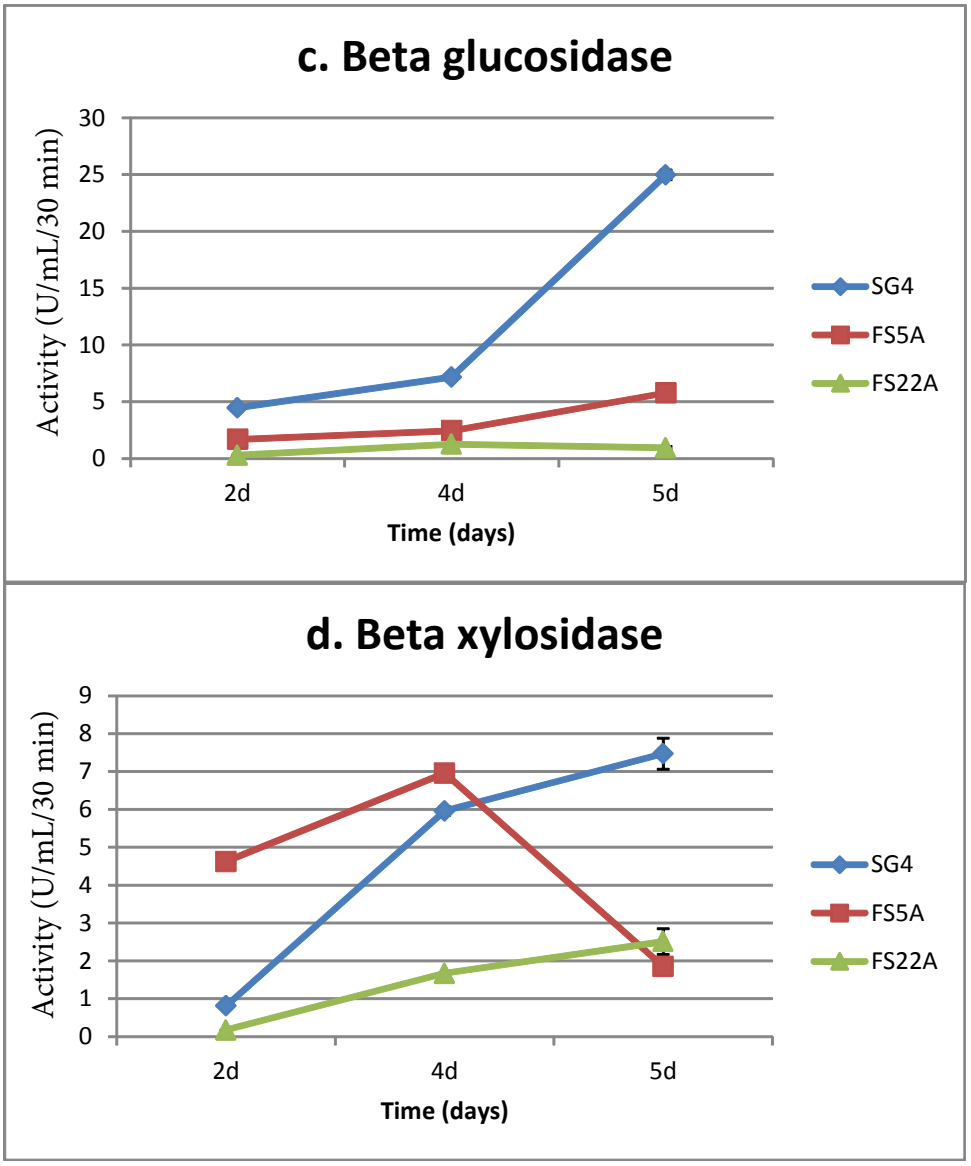


Figure 19 a-d Enzyme activities of *Trichoderma* SG4, *Fusarium* FS5A and FS22A. Mean activities and standard errors indicated.

Figure

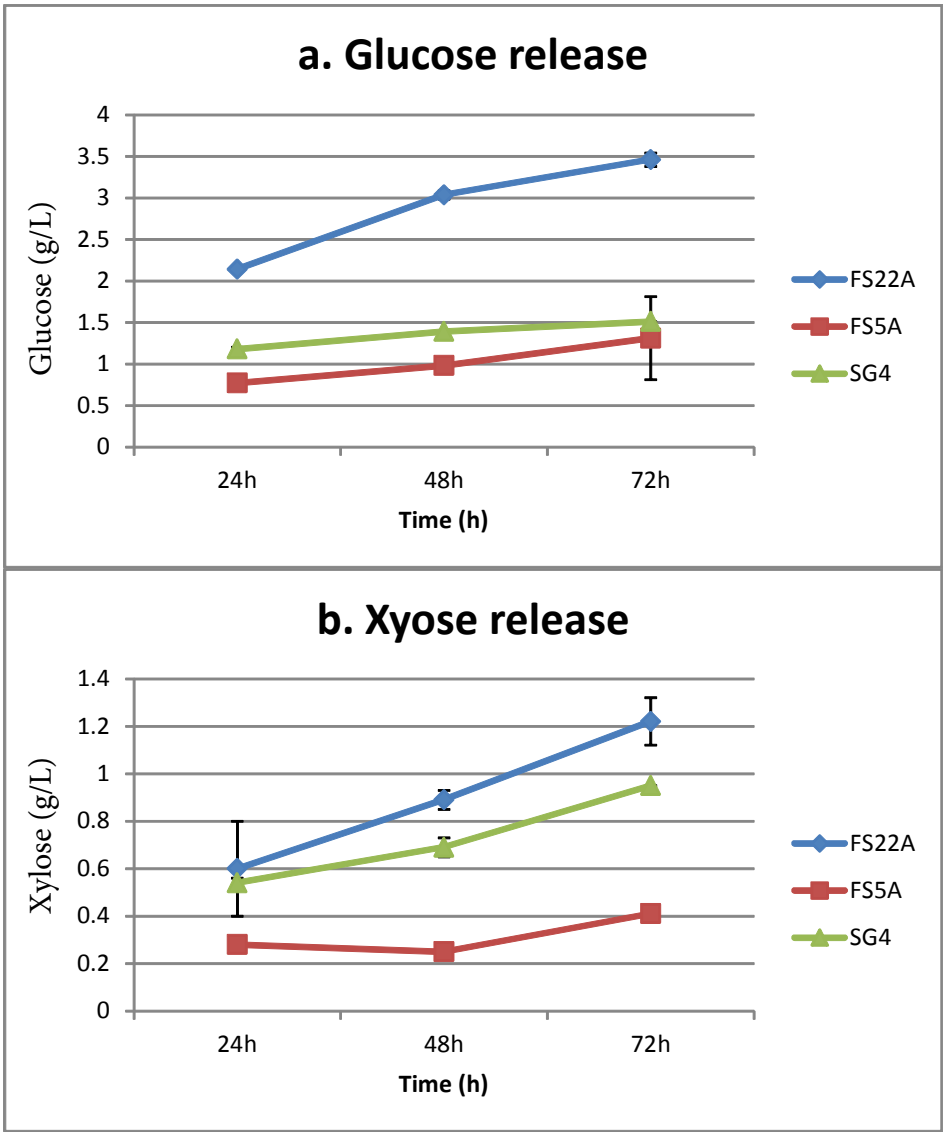


Figure 20 Saccharification of switchgrass FS22A, FS5A and SG4

Figure

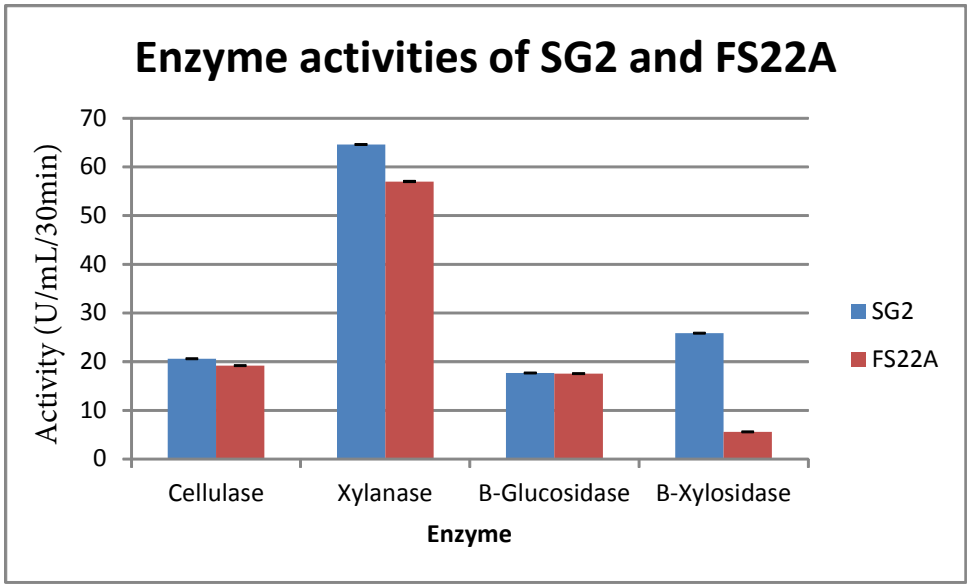


Figure 21 Enzyme activities of SG2 and FS22A. Mean activities and standard errors indicated.

Figure

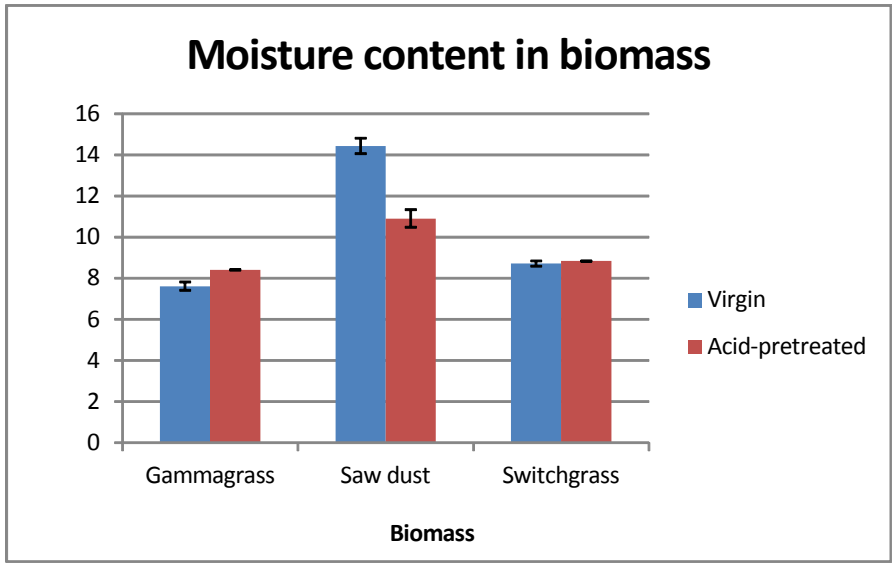


Figure 22 Moisture content in virgin and acid-pretreated biomass

Figure

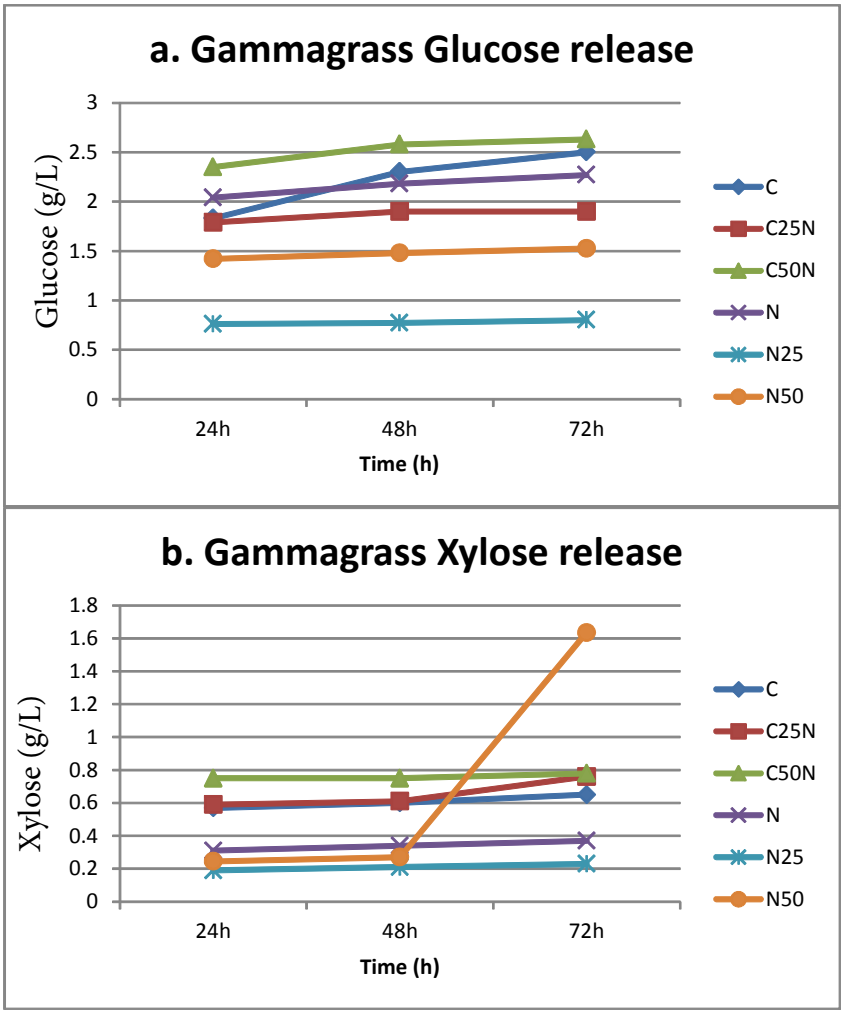


Figure 23 Gammagrass saccharification by *Fusarium* FS22A

Figure

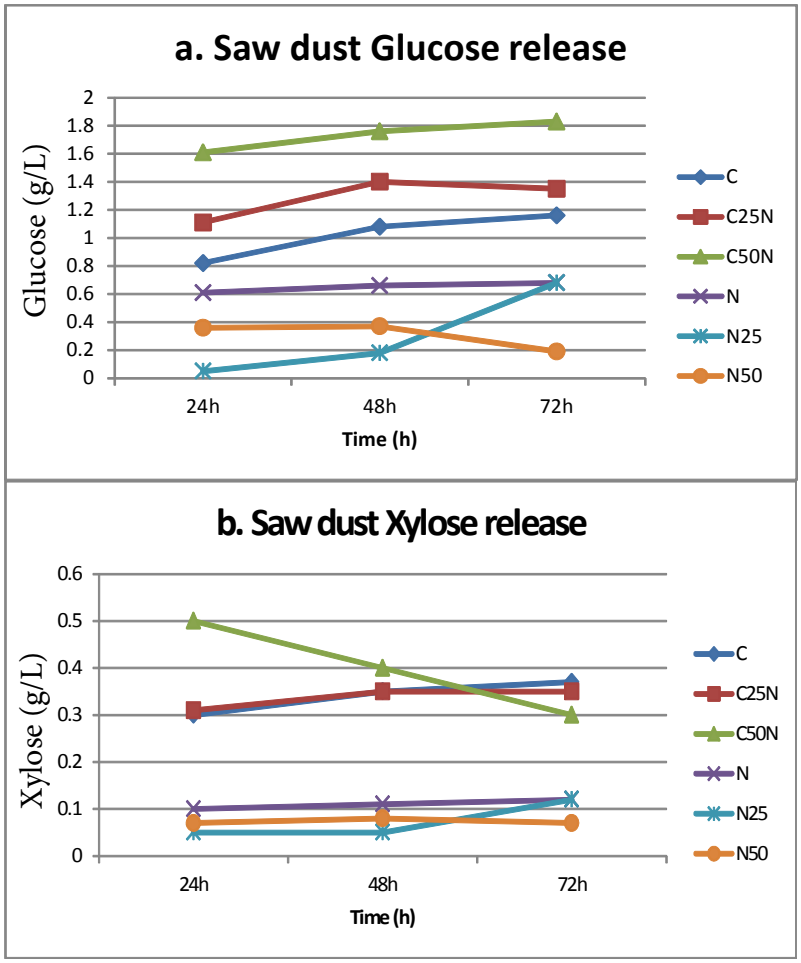


Figure 24 Saw dust saccharification by *Fusarium* FS22A

Figure

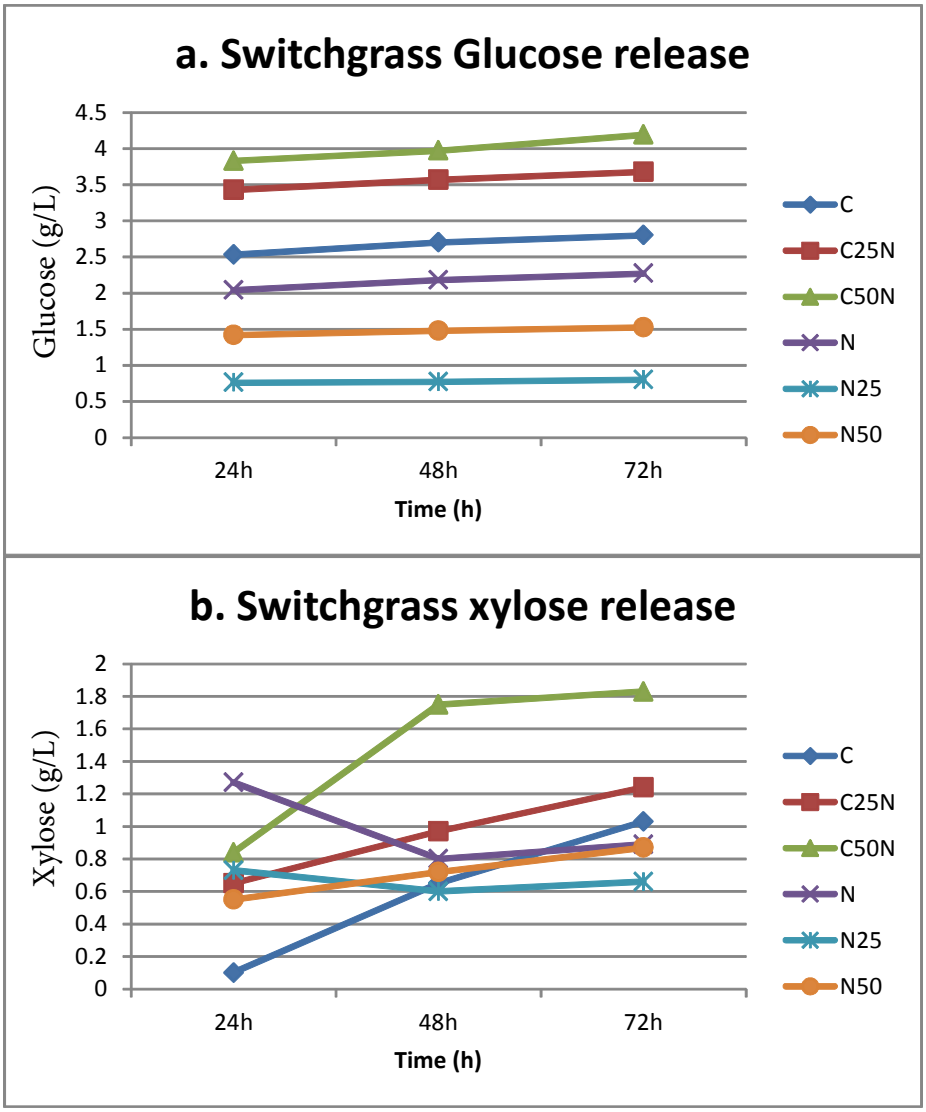


Figure 25 Switchgrass saccharification by *Fusarium* FS22A

Figure

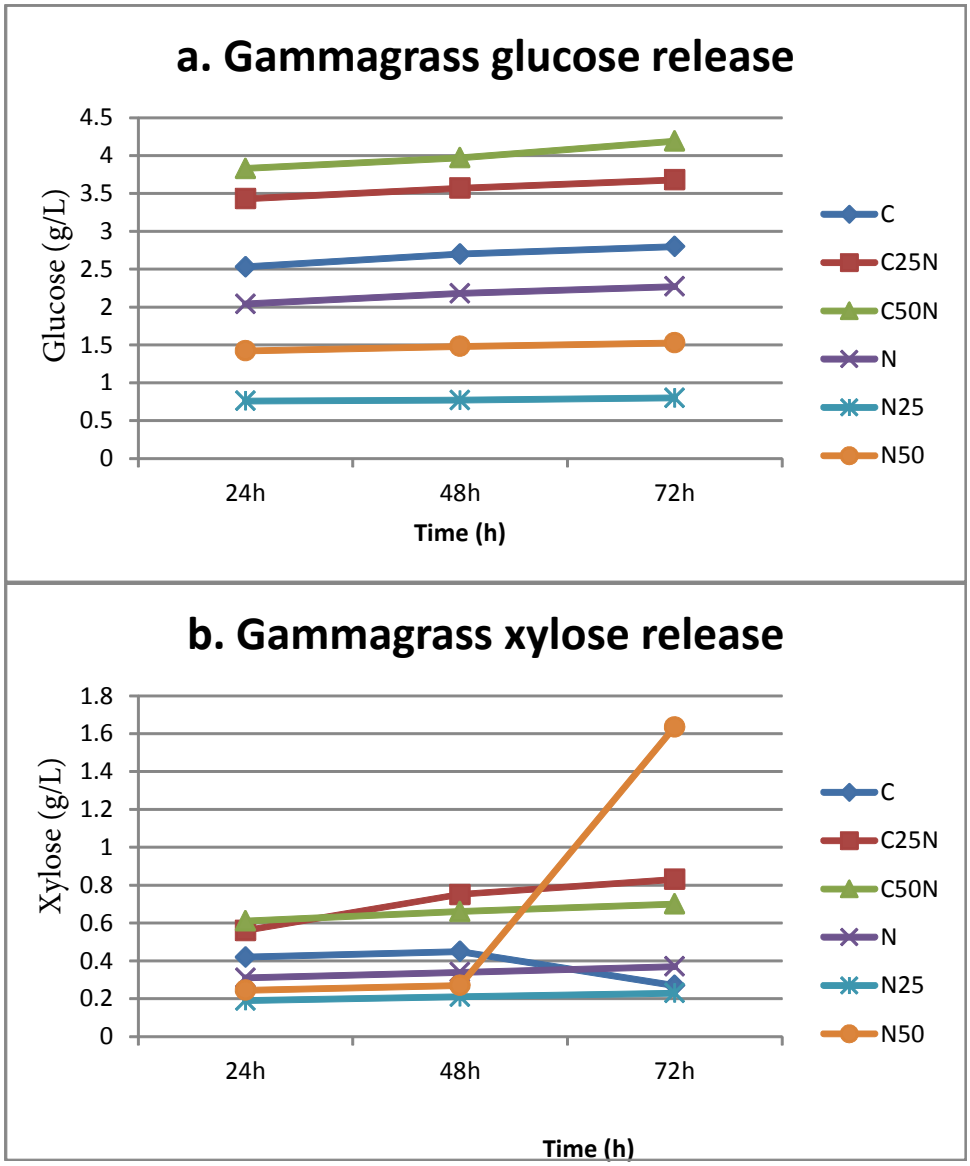
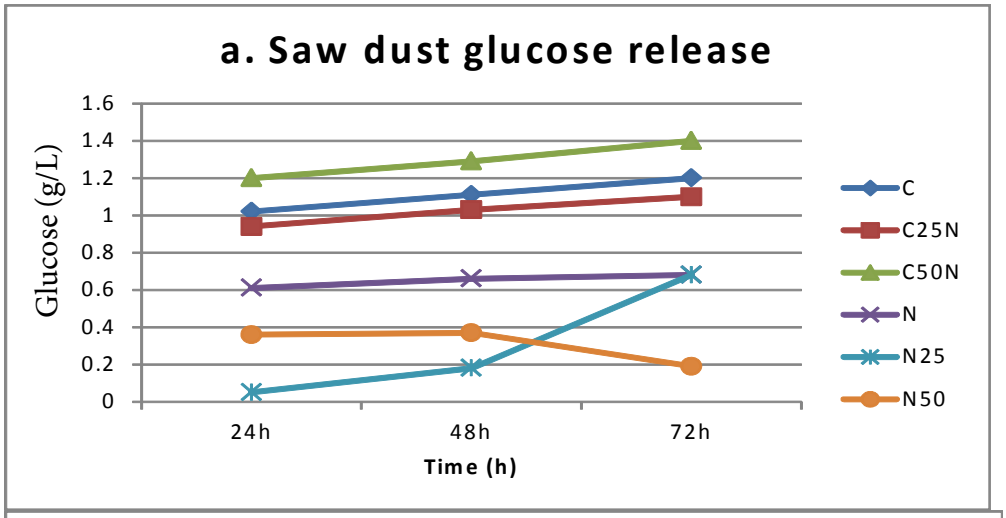


Figure 26 Gammagrass saccharification by *Trichoderma* SG2

Figure



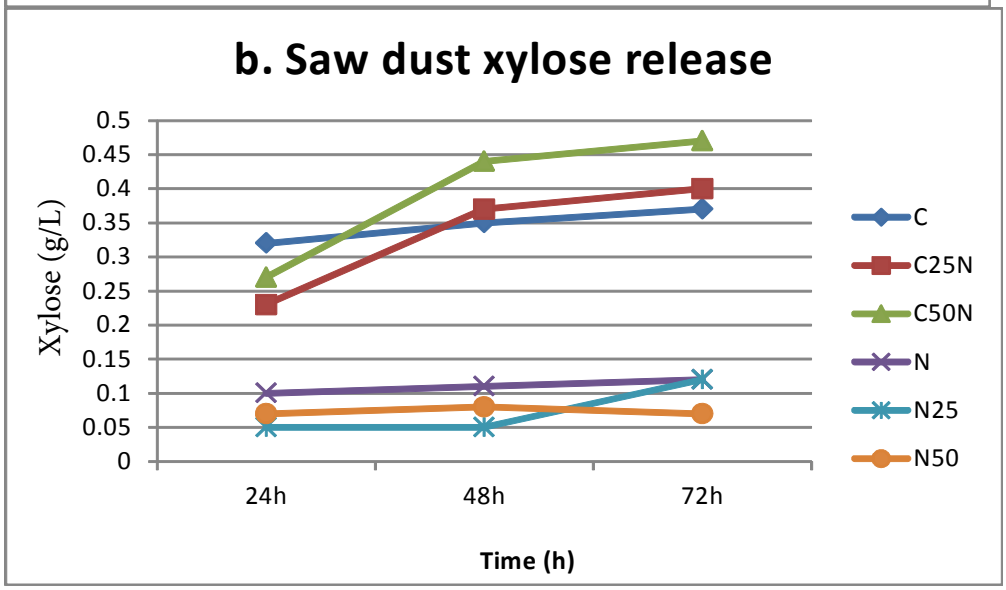


Figure 27 Saw dust saccharification by *Trichoderma* SG2

Figure

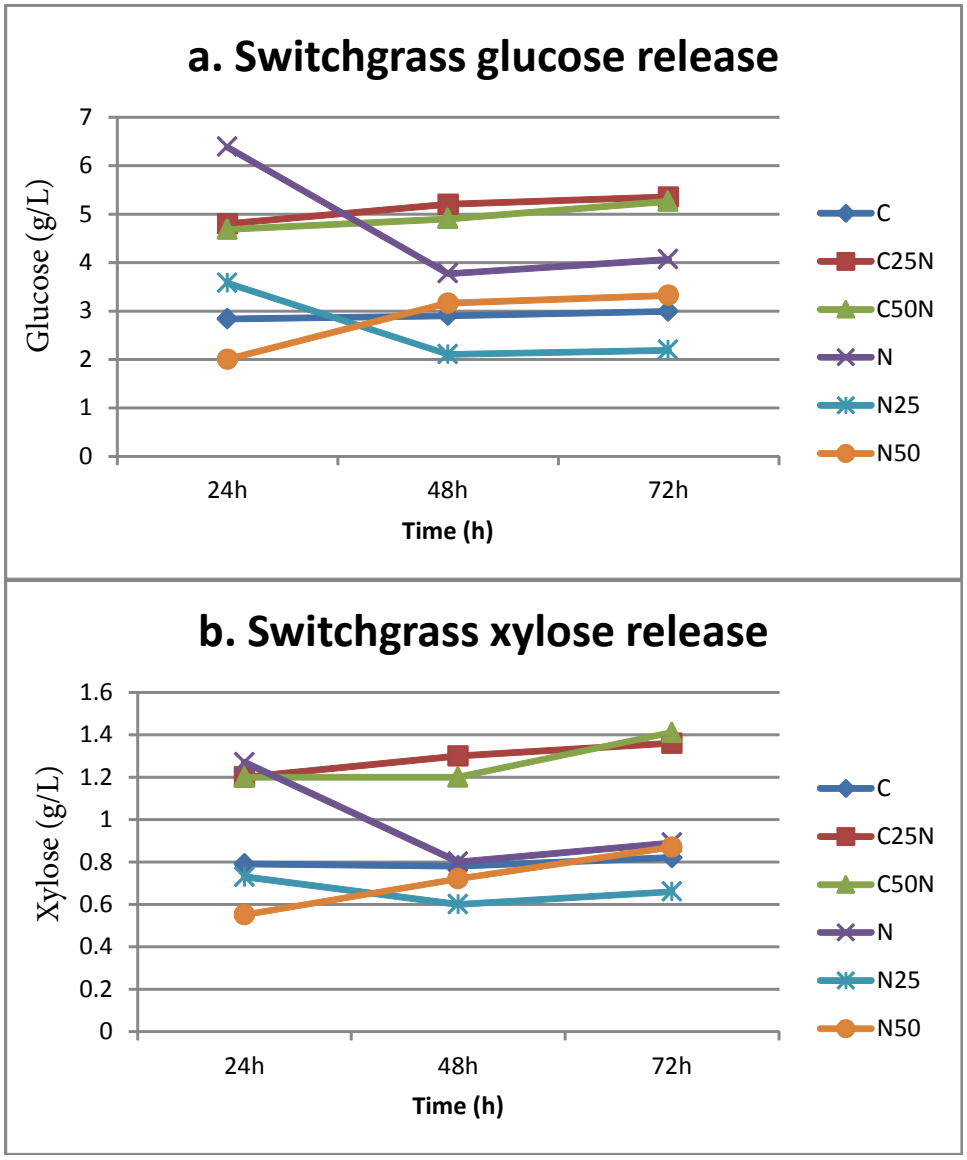
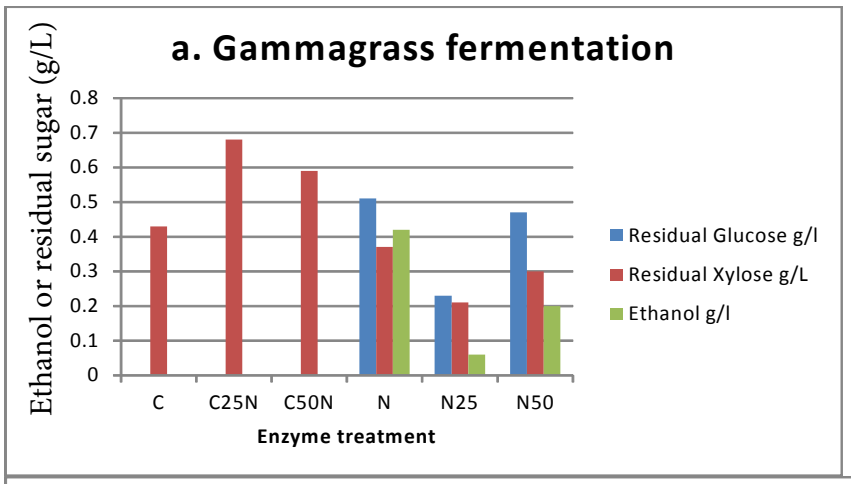
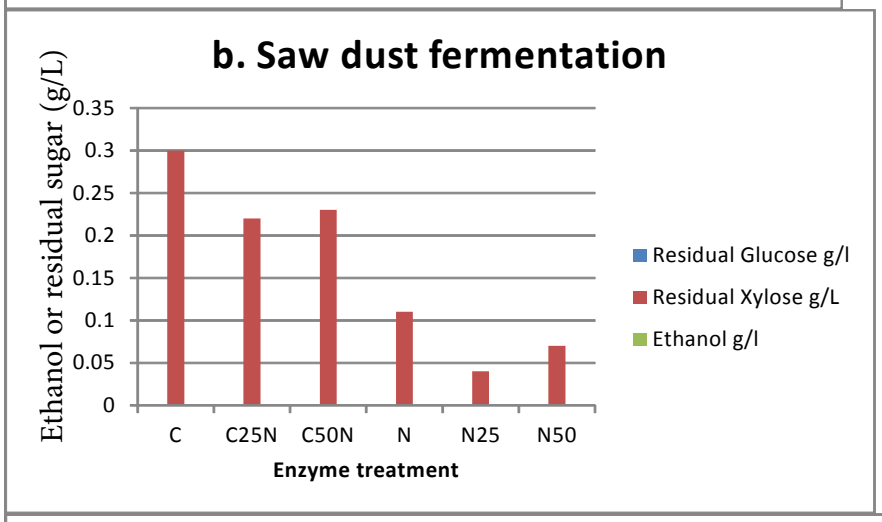


Figure 28 Switchgrass saccharification by *Trichoderma* SG2

Figure





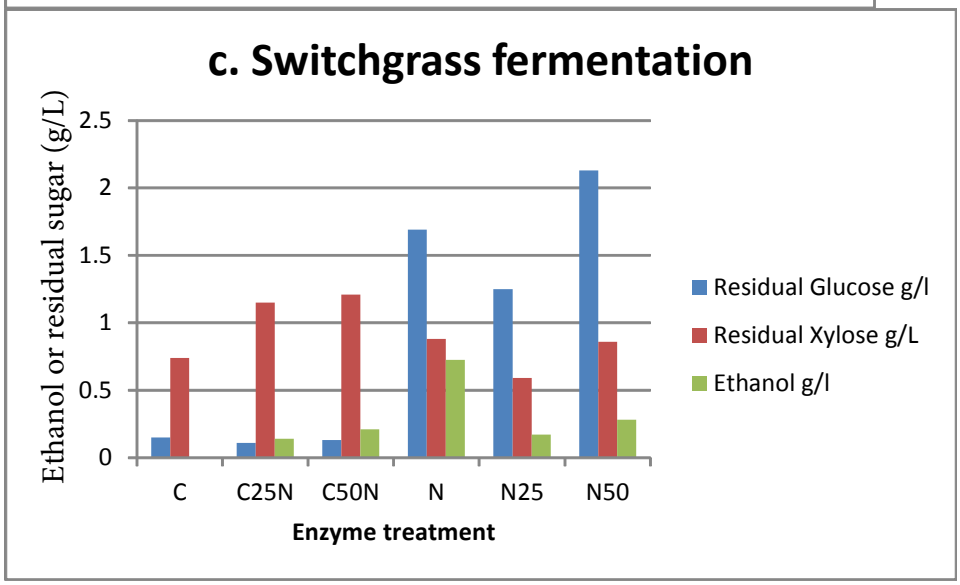


Figure 29 Ethanol fermentation of biomass saccharified by SG2. 72h saccharified biomass was used and 24h fermentation data are presented. a. gammagrass, b. saw dust c. switchgrass

Figure

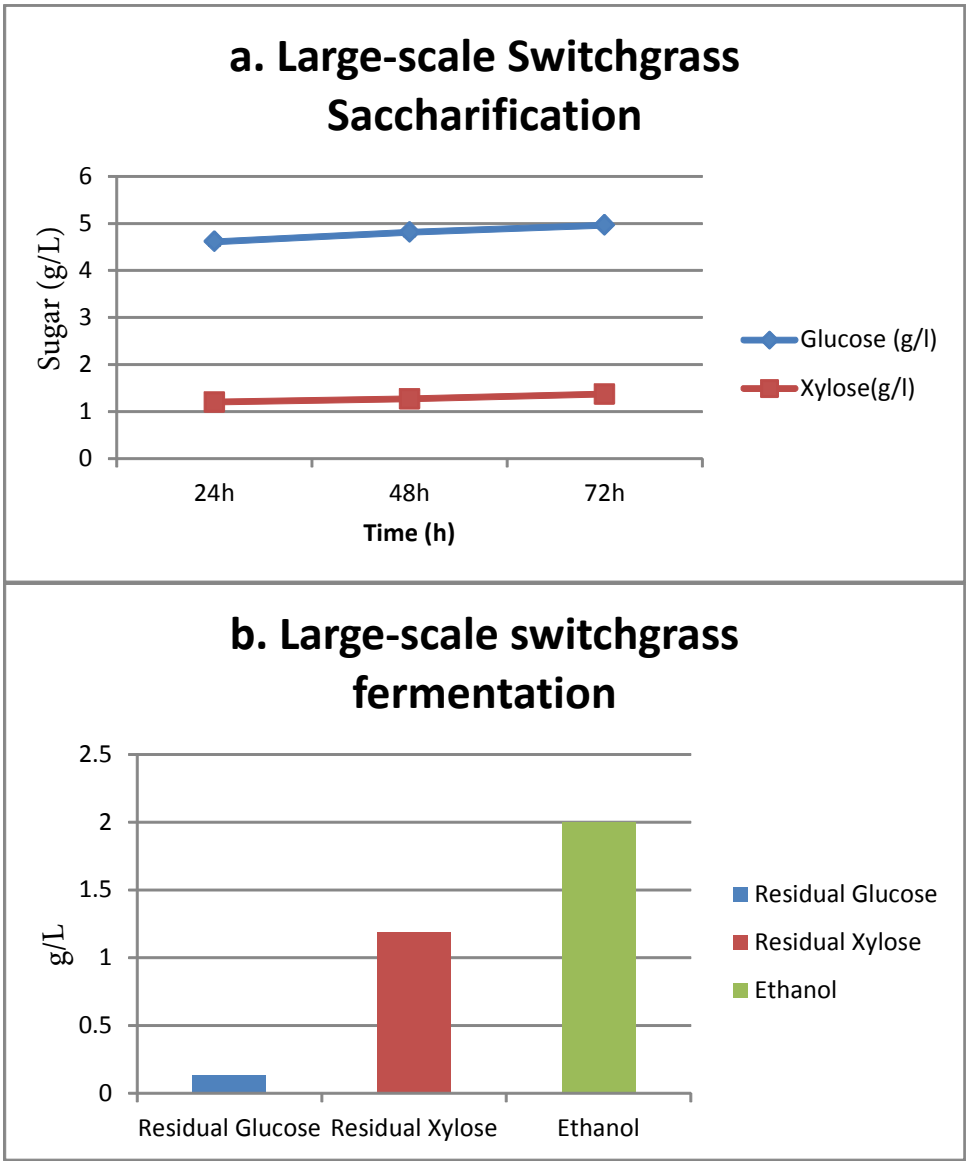


Figure 30 Scale switchgrass saccharification and fermentation by C50N of SG2. a. Saccharification b. 24h Ethanol fermentation of 72h-saccharified switchgrass.

Conclusions and Milestones:

Effect of media volume on production of biomass-saccharifying-enzymes by *Trichoderma* SG2:

- Using 100 ml of enzyme production medium (also known as screening medium) in a 250ml flask compared to 75 mL and 50 mL does not negatively impact enzyme production and can be employed to generate higher volume of the necessary saccharification-enzymes by SG2.

Stability of *Trichoderma* SG2 enzyme activity:

- Routine subculturing of *Trichoderma* SG2 culture does not significantly affect the enzyme activities. SG2 subculturing from agar plates stored at 4°C can be safely used for producing saccharifying enzymes without any negative impact on enzyme activities.

Cellulolytic and xylanolytic enzyme production and saccharification by *Trichoderma* SG4, *Fusarium* FS5A and *Fusarium* FS22A:

- Of the three fungal isolates screened for enzyme activities, it was found that FS22A was the best resulting in 3.5g/L of glucose and 1.22g/L xylose respectively after 72h of saccharification of 20 g/L acid-pretreated switchgrass. It is interesting to note that FS22A identified to be a *Fusarium* sp. produces abundant celluloytic and xylanolytic enzymes and can be a potential source of enzymes in lignocellulosic ethanol production.

Saccharification of biomass by crude enzymes of *Trichoderma* SG2 and *Fusarium* FS22A:

- Although *Trichoderma* SG2 and *Fusarium* FS22A showed similar enzyme activities, SG2 had marginally higher activities than FS22A. Therefore, FS22A also has great potential for use in lignocellulosic ethanol production. Not all *Fusarium* sp. are plant pathogens. In fact addition of nonpathogenic *Fusarium oxysporum* Fo47 to the soil can control tomato foot and root rot (TFRR) caused by pathogenic *Fusarium oxysporum* f. sp. *radicis-lycopersici* by competing for nutrients and attachment sites and also inducing systemic resistance (Bolwer et al 2005). If our isolate FS22A is indeed a nonpathogenic *Fusarium* sp. then it can not only be used in saccharifying lignocellulosic material for ethanol production but also the spent material from enzyme production can potentially be used for microbial plant protection.
- *Trichoderma* SG2 and *Fusarium* FS22A crude aqueous enzyme extracts and in combination with 50% of Novozyme recommended dosage can be used for saccharifying switchgrass for biomass ethanol production.
- Among the three acid-pretreated biomass namely switchgrass, gammagrass and sawdust, switchgrass yielded the highest sugar when crude enzymes from SG2 or FS22A and their combinations with commercial enzyme were used and will be the choice of substrate for further testing.

- Supplementing SG2 or FS22A crude aqueous enzyme extract with 50% or 25% (**C50N** or **C25N**) of commercial enzyme results in the highest sugar yield from switchgrass, suggesting 50-75% potential saving in biomass enzyme.
- In the previous report, ethanol fermentation from acid-pretreated switchgrass samples of 10ml volume in 100ml flasks was reported. The glucose yield after 72h of saccharification was highest in **C50N** with 5.61g/L followed by 5.11g/L in **C25N** (C50N>C25N>N>C). The ethanol yield was the highest in **C50N** at 2.12g/L, 1.7g/L in **C25N**, 1.2g/L in **N** and 1g/L in **C** with ethanol conversion efficiencies of 74%, 65%, 55% and 52% respectively.

Ethanol fermentation in switchgrass using C50N of SG2:

- After 24h fermentation of 1L saccharified sample in 5L bottle, 2.0g/L ethanol was produced with a conversion efficiency of about 80%. At the beginning of fermentation, there was 4.97g/L of glucose and 1.37g/L xylose respectively. Comparably, at the beginning of the ethanol fermentation in 10ml of saccharified sample in 100ml flasks there was 5.26g/L glucose and 1.41g/L xylose respectively. However, the 24h ethanol fermentation in flasks inexplicably yielded only 0.21g/L ethanol with a conversion efficiency of about 4%. In the previous (sixth quarter) report, the acid-pretreated C50N saccharified switchgrass sample of 10ml taken in 100ml flasks yielded 2.12g/L ethanol with a conversion efficiency of 74% and the starting glucose concentration was 5.61g/L and xylose was 1.64g/L respectively.
- Based on the saccharification of acid-pretreated switchgrass samples with different combinations of SG2 crude and commercial enzymes followed by 24h ethanol fermentation in flasks and large media bottles as presented in this and previous (sixth and seventh) reports, it is clear that glucose release of 4.97 to 5.6g/L and xylose release of 1.37 to 1.64g/L is possible from **C50N** leading to ethanol production of 2.0 to 2.12g/L with an ethanol conversion efficiency ranging from 74% to 80%.

Milestone (A.1.ML.1): ***Selected fungi are expected to produce 25g of sugar from 200g of substrate per liter (20% substrate).***

- Our study has achieved 6.34g of total sugars from 20g of substrate per liter which corresponds to **63.4g** of sugars (49.7g glucose and 13.7g xylose) from 200g of substrate. In the case of **flasks**, our study has achieved 6.67g to 7.25g of sugars from 20g of switchgrass substrate that corresponds to **66.7g** to **72.5g** of total sugars from 200g of substrate.
- Previously this milestone was achieved in flasks and this time this milestone has been achieved in scale-up saccharification in lab model bioreactor. This was accomplished by our fungal isolate *Trichoderma* SG2.
- This milestone has also been achieved by another fungal isolate namely, *Fusarium* FS22A in flasks. The crude enzyme extract yielded 5.5g of total sugars (4.41g glucose and 1.03g xylose) from 20g of switchgrass substrate which corresponds to **54.4g** of sugar from 200g of substrate. Furthermore, the crude enzyme of FS22A with 50% commercial enzyme (**C50N**) yielded 8.96g sugar (7.13g glucose and 1.83g xylose) from 20g of substrate which corresponds to about **90g** sugar from

200g of substrate. This however needs to be validated in scale-up saccharification in lab model bioreactor.

Milestone (B.1.ML.1): ***Selected yeasts are expected to produce 45g of ethanol from 100g of xylose or glucose per liter.***

- Our study has achieved 2.0g of ethanol from 4.97g of glucose using *S. cerevisiae* which corresponds to **40g** of ethanol from 100g of glucose. In the case of **flasks**, 2.12g ethanol was obtained from 5.61g of glucose which corresponds to about **38g** of ethanol from 100g of glucose.
- Since our ethanol production is very close to the target, process optimization is likely to achieve the target.
- Our new yeast isolates underperformed in xylose conversion to ethanol and will be subjected to optimization in future studies.

Milestone (C.1.ML.1): ***Determine most efficient substrate for conversion to ethanol. Microbial consortium/enzyme or enzymes are expected to produce about 25 g of sugar (C5 and C6 sugars) from about 200 g of substrate per liter.***

- Previously, it was found that acid-pretreated switchgrass yielded the maximum sugars and ethanol when compared with virgin, autoclaved and alkali-pretreated switchgrass.
- In this report we found that acid-pretreated switchgrass yielded the maximum sugars when compared to acid-pretreated gammagrass and acid-retreated sawdust. Therefore, it is ascertained that acid-pretreated switchgrass is the best choice for saccharification and ethanol production.
- A consortium of microbial enzymes (crude aqueous extract along with 50% and 25% commercial enzymes and commercial enzyme full strength, 50% and 25% only) from two of the most promising fungal isolates namely *Trichoderma* SG2 and *Fusarium* FS22A were evaluated for sugar release from three different substrates.
- **C50N** was the best combination in both SG2 and FS22A for sugar release after 72h saccharification in all three substrates. Additionally, **C25N** also released comparable amount of sugar as **C50N** in switchgrass.
- This milestone has been achieved by **C50N** of **SG2** in acid-pretreated switchgrass as it yielded 6.34g of total sugars from 20g of substrate per liter which corresponds to **63.4g** of sugars (49.7g glucose and 13.7g xylose) from 200g of substrate in scale-up saccharification whereas in **flasks**, it produced 6.67g to 7.25g of sugars from 20g of switchgrass substrate that corresponds to **66.7g** to **72.5g** of total sugars from 200g of substrate.
- This milestone has been achieved by **C** and **C50N** of ***Fusarium* FS22A** in acid-pretreated switchgrass. The crude enzyme extract **C** in flasks yielded 5.5g of total sugars (4.41g glucose and 1.03g xylose) from 20g of switchgrass substrate which corresponds to **54.4g** of sugar from 200g of substrate and the crude enzyme of FS22A with 50% commercial enzyme (**C50N**) yielded 8.96g sugar (7.13g glucose and 1.83g xylose) from 20g of substrate which corresponds to about **90g** sugar from 200g of substrate. This however needs to be validated in scale-up saccharification in lab model bioreactor.

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PART 7: Research Activities of FY-12 (July 1, 2012 to September 30, 2012).
Written by: Benedict Okeke, and Ananda Nanjundaswamy

Introduction:
The data from the previous quarter showed that milestones outlined for tasks A, B and C were achieved. The milestone for task C ie., achieving 45g ethanol from 100g of glucose or xylose was almost met by producing 40g of ethanol from 100g of glucose. It was also concluded that up to 100ml of screening medium could be used in a 250ml flask without impacting enzyme yield. Routine subcultuing of *Trichoderma* SG2 did not affect enzyme activities. *Trichoderma* SG2 and *Fusarium* FS22A had similar enzyme activities and supplementing them with 25% or 50% of recommended commercial enzyme yielded the highest sugars from switchgrass of the three feedstocks evaluated namely switchgrass, gammagrass and sawdust. In this quarter the following objectives were addressed:

Specific Objectives
1. Effect of switchgrass particle size on saccharification by crude *Trichoderma* SG2 enzyme
2. Effect of switchgrass feedstock concentration on saccharification by crude *Trichoderma* SG2 enzyme
3. *Trichoderma* SG2 saccharification of waste paper powder
4. Effect of paper powder supplementation to switchgrass feedstock on sugar yield by crude SG2 enzyme
5. Sequential enzyme addition to saccharified-switchgrass to obtain maximum sugar yield
6. Effect of SG2 culture age on switchgrass saccharification
7. Scale-up validation of saccharification and ethanol fermentation of switchgrass by *Trichoderma* SG2
8. Compositional analyses of virgin and pretreated feedstock used in this project

Materials Methods:
Effect of switchgrass particle size on saccharification by crude *Trichoderma* SG2 enzyme
Switchgrass was powdered using Fritsch Pulverizer 16 mill to obtain particle sizes of 0.12mm, 0.2mm, 0.25mm, 0.50mm, 0.75mm, 1.0mm, 2mm, 3mm and 4mm. For each particle size, standard US sieves were used during milling. Samples collected were stored in Ziplock bags. Prior to saccharification, samples were acid-pretreated as, air dried at room temperature and stored in Ziplock bags. For acid treatment, ACS grade H_2SO_4 was used. A 2% solution of H_2SO_4 was prepared using tap water. Approximately 10g of switchgrass was weighed into a 250ml flask and 90 ml of 2% H_2SO_4 was added and the mouth of the flask was covered with an aluminum foil. Duplicate flasks were prepared and autoclaved at 121^0C for 60 min. After autoclaving flasks were carefully removed and strained using a muslin cloth to remove liquid portion. A small sample of the liquid was collected for analysis of chemical composition of metabolites during acid hydrolysis. The solid portion (pretreated switchgrass) was washed several times to remove acid. The complete removal of the acid was confirmed by suspending a small portion of the sample in water and pH measured.

Saccharification
Saccharification was carried out in 100ml flasks with airtight caps. About 10ml of SG2 crude enzyme extract was taken in each flask. About 2% of acid-pretreated switchgrass was added to each flask. All experiments were carried out in duplicates. Saccharification was carried out at 50°C at 80rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All the flasks were closed tightly to avoid escape of moisture. About 0.5ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Enzyme production medium:
Crude enzyme production was carried out in 250ml flasks containing optimized screening medium. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution). Each flask contained 100ml medium and flasks sterilized at 121°C for 1h before inoculation. About 10% of 72h inoculum was added into each flask aseptically, incubated at 30°C for 5 days. The crude enzyme samples obtained on day 5 was assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase activities. On 5^{th} day of enzyme production, the broth was transferred into sterile 50ml centrifuge tubes and centrifuged at 5000rpm for 10 min. The supernatant was used for biomass saccharification.

Effect of switchgrass feedstock concentration on saccharification by crude *Trichoderma* SG2 enzyme
Acid-pretreated switchgrass was used at concentrations of 2, 5, 10, 15 and 20% and the experiment was carried out as outlined above.

Effect of SG2 saccharification of paper powder
Office waste (white, printed) shredded paper was milled and stored in Ziplock bags. Acid pretreatment was carried out as described above. Steam pretreatment was carried out using Sanyo vertical autoclave using a 60 min cycle at 121°C. In this experiment, virgin (un-treated) paper powder at 2% and 5%, steam pretreated and acid-pretreated samples were used for saccharification. Enzyme production, saccharification and sugar estimation were carried out as described above.

Effect of paper powder supplementation to switchgrass feedstock on sugar yield by crude SG2 enzyme
Feedstock at 2, 4, 5 and 6% were used with 50:50 of acid-pretreated switchgrass and acid-pretreated paper powder. Enzyme production, saccharification and sugar estimation were carried out as described above.

Sequential enzyme addition to saccharified switchgrass to obtain maximum sugar yield
SG2 crude enzyme was produced as described above. Acid-pretreated switchgrass at 2% and 5% concentrations were used. Experiment was carried out in duplicates. Samples were treated with enzyme and after 24h the sample was centrifuged at 14000*g* for 15 min using Beckman Coulter centrifuge, supernatant removed and collected for sugar analysis and fresh crude enzyme sample was added to the saccharified residue, transferred to flask and allowed to saccharify for another 24h. Repeated extraction was conducted so as to obtain data after 24h, 48h and 72h. Enzyme production, saccharification and sugar estimation were carried out as described above.

Effect of SG2 culture age on switchgrass saccharification
Two month SG2 culture grown on potato dextrose agar, stored at 4 deg C was used to inoculate seed flask and enzyme production was carried out as described above. For new SG2 enzyme production, freshly growing (5-6d old) plate was used to inoculate seed medium and enzyme production was carried out as described.

Scale-up validation of saccharification and ethanol fermentation of switchgrass by *Trichoderma* SG2

Enzyme production:

Crude enzyme extract of *Trichoderma* SG2 was produced in ten 250ml flasks using optimized screening medium. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution). Each flask contained 100ml medium and flasks were sterilized at 121°C for 1h before inoculation. About 2% of 72h inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5th day the broth was transferred into sterile 250ml centrifuge tubes, centrifuged at 4000 rpm for 20 min, supernatant pooled from all tubes, and approximately 1000 ml of crude enzyme extract was used for saccharification study. The crude enzyme samples were assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase.

Saccharification:

Saccharification was carried out at 50°C in a 5L glass media bottle with airtight cap. About one liter of the SG2 crude enzyme extract was taken in the bottle to which 50g of acid-pretreated switchgrass (5% feedstock) was added. Periodically samples were collected at 24, 48 and 72h and analyzed for sugar release.

Ethanol fermentation:

After 72 h, 100 µl of 48 h old *Saccharomyces cerevisiae* inoculum which was washed in sterile water and re-suspended in sterile water was added and the 5L glass media bottle was incubated at 30°C on a shaker at 65 rpm. Samples were drawn at 24 h, 48 h and 72 h to quantify sugar release and ethanol production. This experiment was repeated twice.

Compositional analyses of virgin and pretreated feedstock used in this project

About 50 g of duplicate samples of feedstock-virgin and acid-pretreated switchgrass (old and new), gammagrass and sawdust were analyzed for crude fiber (AOAC Official Method 978.10, 2006), acid-detergent fiber (AOAC Official Method 973.18 (A-D), 2006), neutral detergent fiber (JAOAC 56, 1352-1356, 1973), cellulose (AOAC Official Method 973.18 (A-D), 2006.), hemicellulose (NDF – ADF), lignin (AOAC Official Method 973.18 (A-D), 2006.), protein (Combustion Analysis (LECO) AOAC Official Method 990.03, 2006; Kjeldahl, AOAC Official Method 984.13 (A-D), 2006; Standard report of 'crude protein' utilizes the calculation: 6.25 X Nitrogen value) and ash (AOAC Official Method 942.05) at the Agricultural Experimental Station Chemical Laboratories, University of Missouri, Columbia, MO.

Statistical analyses:

Statistical Analysis Software (SAS, version 9.3) was used for data analyses. When required, Analysis of Variance (ANOVA) was used to compare treatments. PROC GLM was used and pair-wise differences were ascertained using *Tukey* correction at $P=0.05$.

Results and Discussion

Effect of switchgrass particle size on saccharification by crude *Trichoderma* SG2 enzyme

The effect of particle size on glucose and xylose yield was significant ($P<0.0001$) but there was no conceivable trend. For example, the following trend was seen with glucose production 3mm>4mm>0.5mm>2mm>1.5mm>0.12mm>0.25mm>1mm>0.2mm>0.75mm. Similarly, the effect of particle size on xylose production was significant but with a more plausible trend

as follows 2mm>3mm>4mm>0.5mm>1.5mm>0.12mm>0.2mm>1.0mm>0.75mm. The analyses of each particle size showed that the sugar yield (glucose or xylose) does not significantly vary over time (Fig. 1a, b).

Effect of switchgrass feedstock concentration on saccharification by crude *Trichoderma* SG2 enzyme

The effect of five different concentrations of switchgrass on saccharification by SG2 crude is outlined in Fig 2a and 2b. Overall, effect of feedstock concentration had a significant effect ($P<0.0001$) with 10%>5%>2%>20%>15% and time was significant 72h>48h>24h for glucose yield. Within each concentration, glucose yield increased over time significantly for 5%, 10% and 15% only. In the case of xylose, the different concentrations were not significantly different ($P=0.1451$) whereas time was significant with 72h>48h>24h, ie., with time the sugar yield increased. The highest glucose yield was **15.8g/L** and xylose yield was **3.8g/L** from **10% switchgrass** after 72h.

Effect of SG2 saccharification of paper powder

Only **acid-pretreated 5% paper powder** yielded the highest glucose concentration of **10.3g/L** after 48h starting with 8.5g/L at 24h whereas the highest xylose concentration **3.9g/L** at 48h starting with 2.7g/L at 24h (Fig. 3). The sugar yields at 72h were not any different from that at 48h. Sugar yields in autoclaved paper-powder, 5% and 2% virgin, un-pretreated paper powder were negligible with less than 0.5g/L after 24h and no sugars were detected after 48h and are therefore not represented graphically.

Effect of paper powder supplementation to switchgrass feedstock on sugar yield by crude SG2 enzyme

The effect of supplementation of paper powder to switchgrass at 50:50 to achieve total concentration of 2%, 4%, 5% and 6% are outlined in figs 4a and b. At 6% concentration of feedstock (paper powder: switchgrass), the highest sugar, both glucose and xylose yields were obtained at $P<0.0001$ and the trend was 6%>5%>4%>2%. With increasing time, sugar yield also increased significantly at $P<0.0001$ (72h>48h>24h yield) for both glucose and xylose. Analysis of each concentration showed that the glucose and xylose yields did not vary significantly with time for 2, 4, and 5% whereas it was significant for 6% with highest yield at 72h ($72h^{a}>48h^{b}>24h^{b}$ for glucose and $72h^{a}>48h^{a}>24h^{b}$ for xylose). The highest glucose yield of **15g/L** and xylose yield of **4.4g/L** after 72h saccharification was obtained from **6% feedstock**.

Sequential enzyme addition to saccharified switchgrass to obtain maximum sugar yield

The first enzyme addition resulted in 6.26 and 5g/L glucose and 1.9 and 1.6g/L xylose from 2% and 5% switchgrass respectively after 24h of first saccharification(Figs. 5a, b). Addition of SG2 crude enzyme ie., second saccharification of the same biomass resulted in 1.4 and 5 g/L glucose and 0.4 and 1.6g/L xylose respectively from 2% and 5% switchgrass and the third saccharification resulted in 0.05 and 0.3g/L glucose and 0 and 0. 8g/L xylose from 2% and 5% switchgrass respectively. Therefore after repeated extraction at the end of 72h, a total of **7.5** and **10.3g/L glucose** and **2.3 and 3.9g/L xylose** were obtained from **2% and 5% switchgrass** respectively.

Effect of SG2 culture age on switchgrass saccharification

Enzyme age did not affect the sugar yields irrespective of enzyme age (Figs. 6a, b). Also the yields did not vary over time for 2, 5 and 10% substrate as far as glucose was concerned and it varied over time for 10% and 2% for xylose. The enzyme activities of old and new SG2

culture are provided in Table 1. There was no significant difference in enzyme activity profile between old and new culture.

Scale-up validation of saccharification and ethanol fermentation of switchgrass by *Trichoderma* SG2

Using **5%** acid-pretreated switchgrass, **6.4g/L glucose** and **1.7g/L xylose** was obtained after 72h saccharification by SG2 enzyme (Fig. 7). The sugar yields did not vary statistically over time for both glucose and xylose production. Ethanol fermentation by *Saccharomyces cerevisiae* resulted in **2.84g** of **ethanol** after 24h.

Compositional analyses of virgin and pretreated feedstock used in this project

The compositional analyses of all the major feedstock (virgin and acid-pretreated) used in this project were undertaken at the Agricultural Experimental Station Chemical Laboratories, University of Missouri, Columbia, MO and is outlined in Table 2.

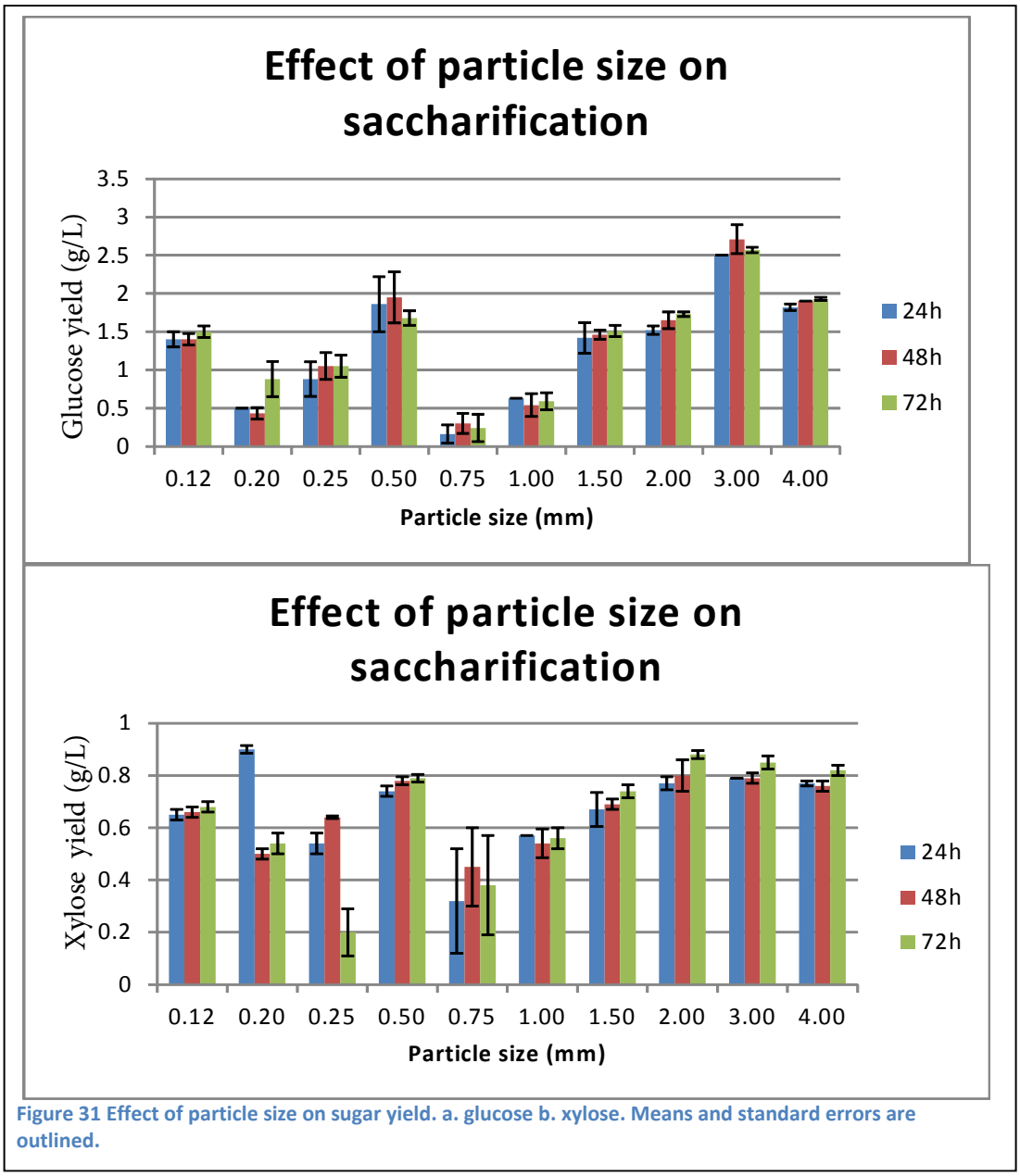
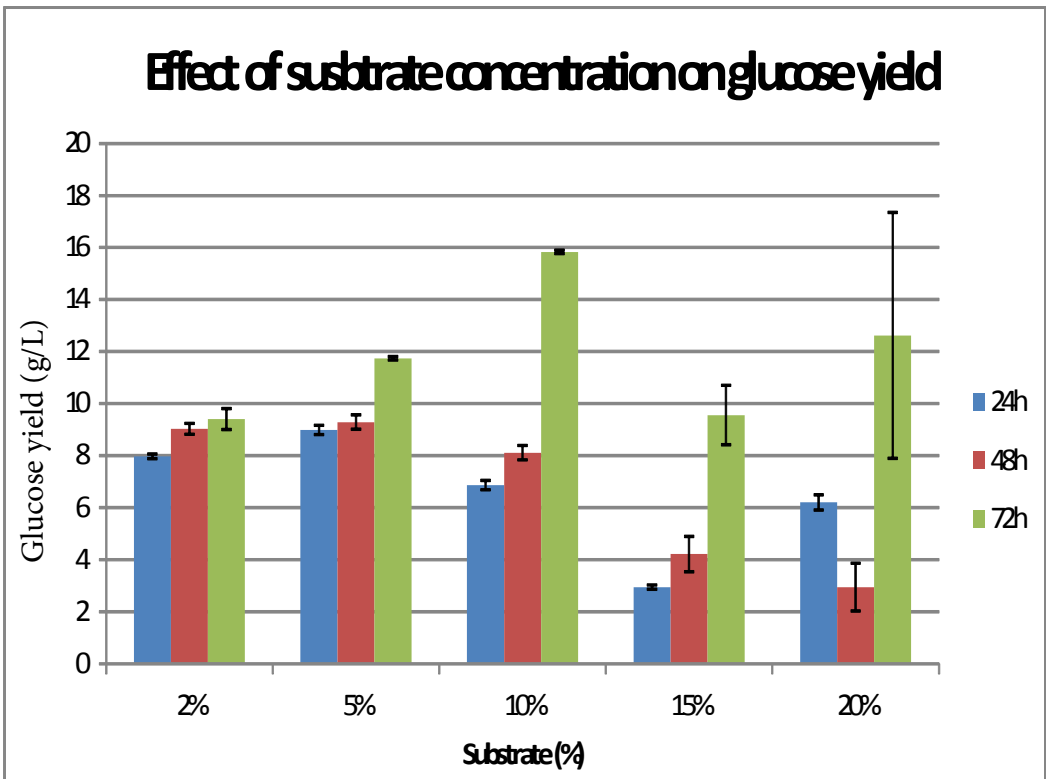


Figure 31 Effect of particle size on sugar yield. a. glucose b. xylose. Means and standard errors are outlined.



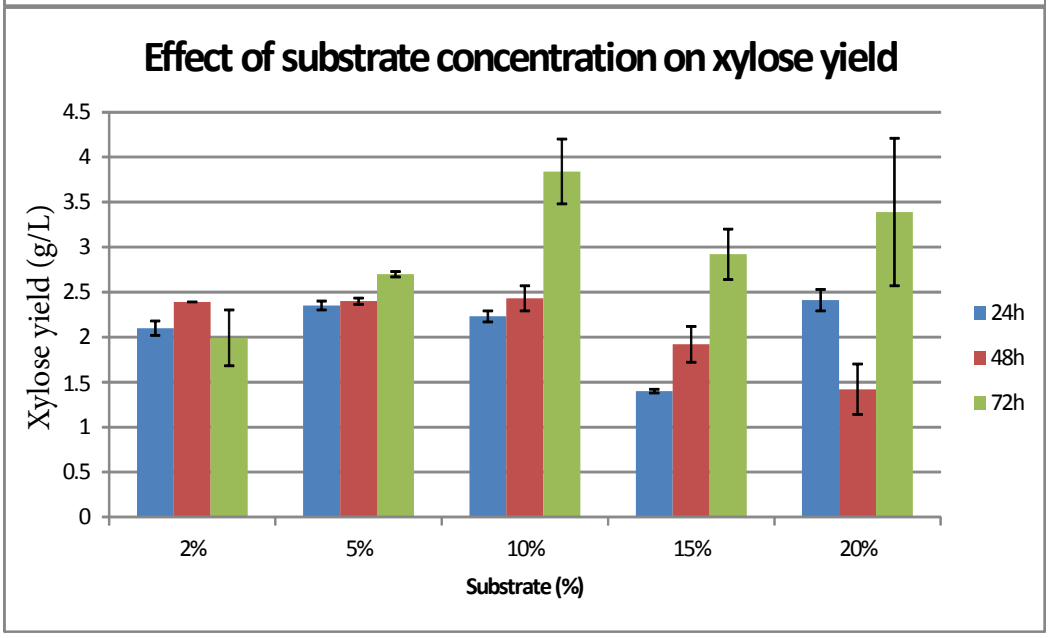


Figure 32 Effect of feedstock concentration on sugar yield. a. glucose b. xylose. Means and standard errors are indicated.

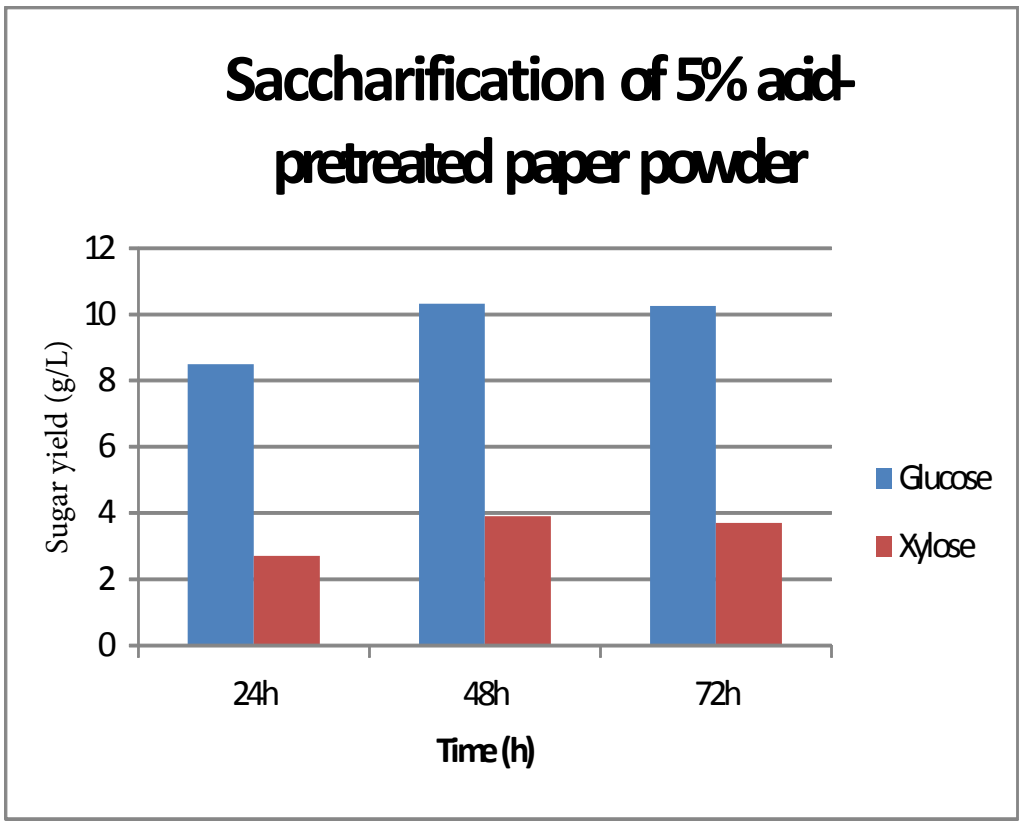
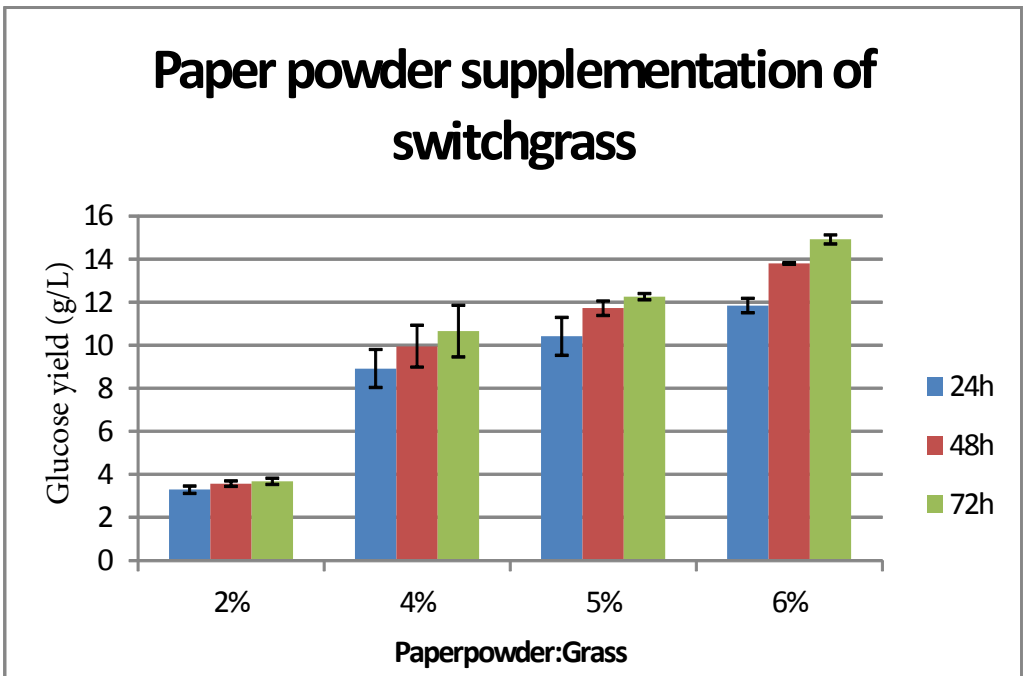


Figure 33 Saccharification of acid-pretreated paper powder.



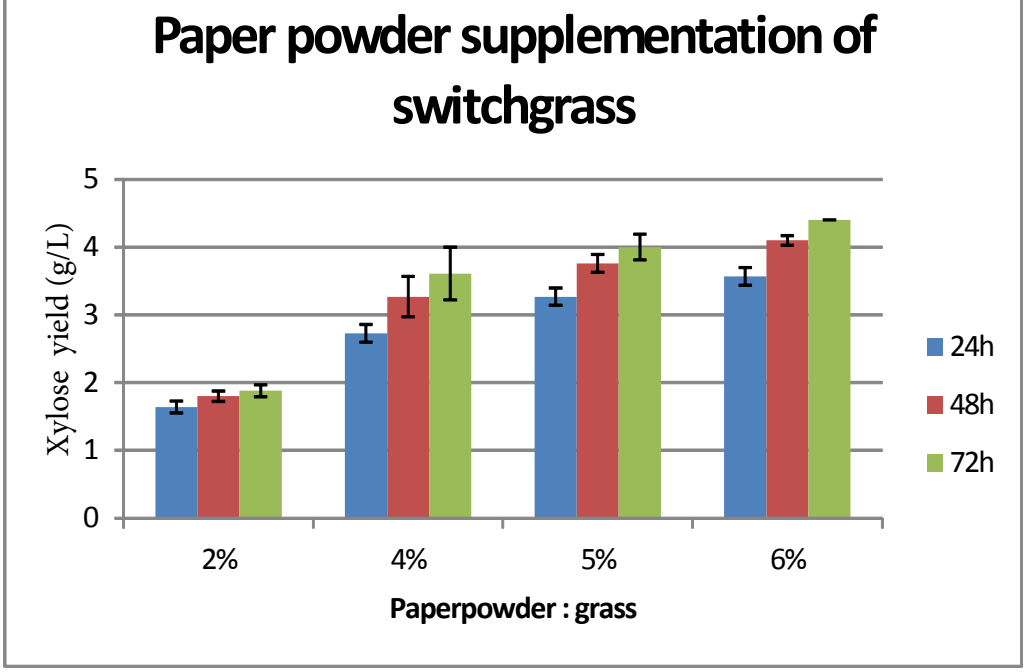


Figure 34 Paper powder supplementation of switchgrass. a. glucose b. xylose. Means and standard errors are indicated. Paper powder:switchgrass=1:1.

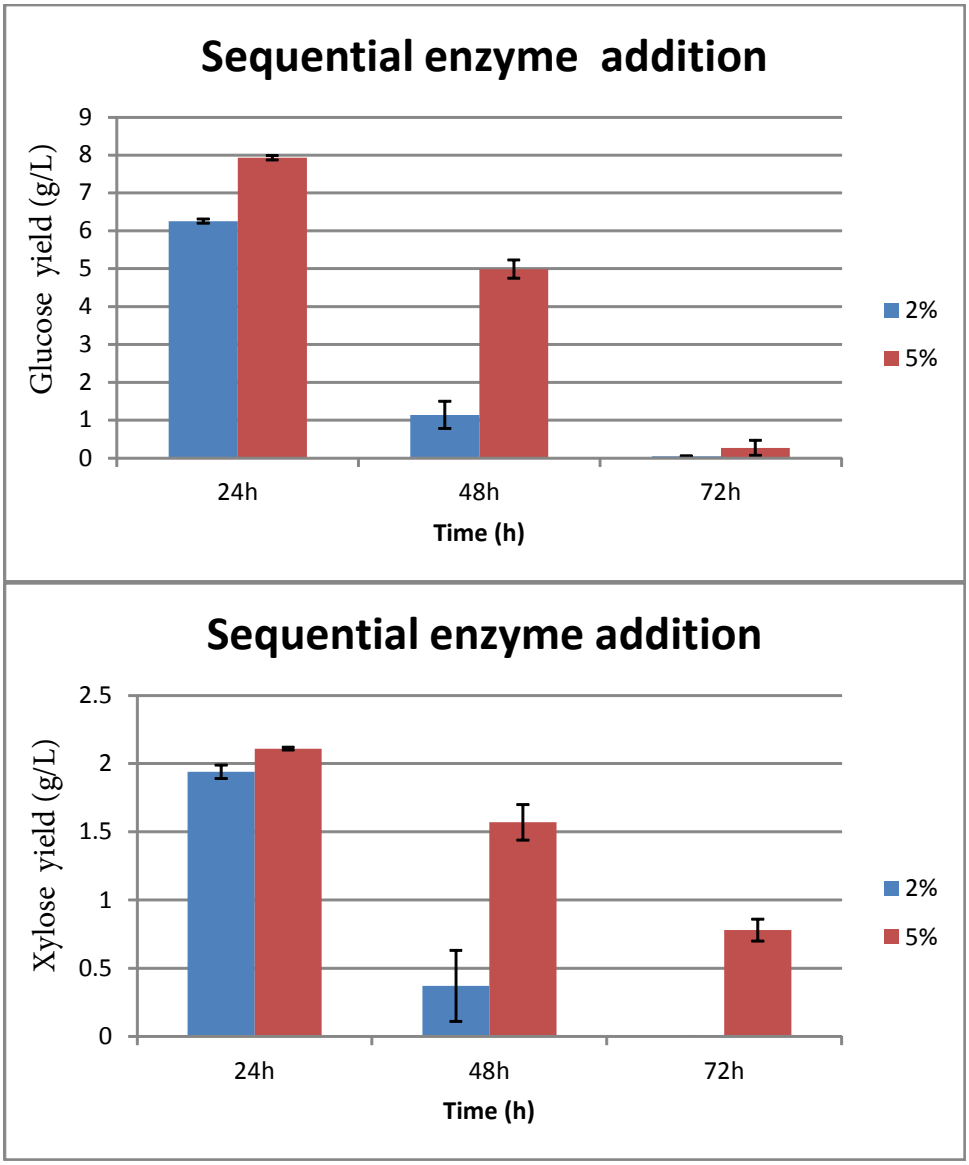


Figure 35 Sequential saccharification of saccharified-switchgrass feedstock. a. Glucose b. xylose. Means and standard errors are indicated.

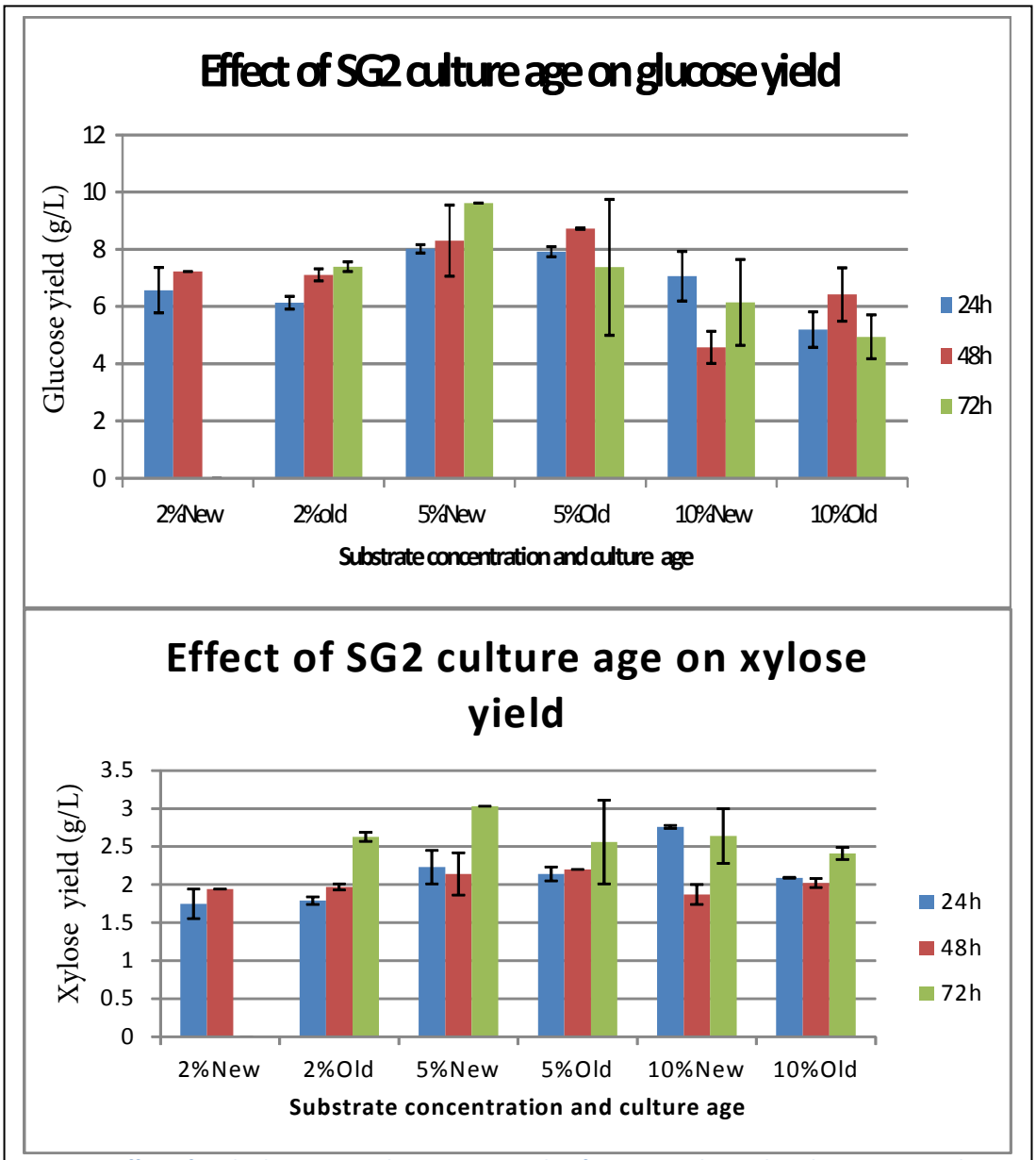


Figure 36 Effect of Trichoderma SG2 culture age on saccharification. a. Glucose b. Xylose. Means and standard errors are indicated.

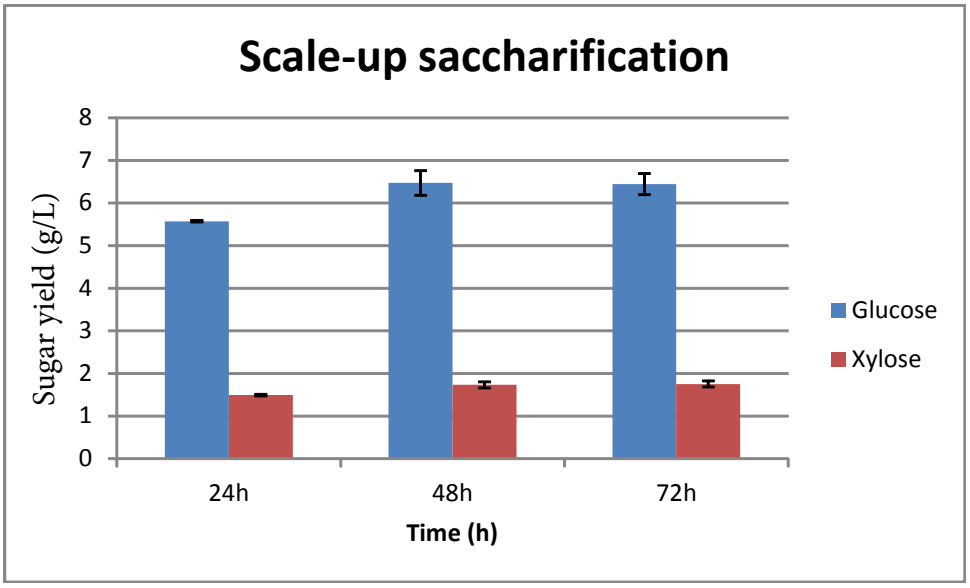


Figure 37 One-liter saccharification of 5% acid-pretreated saccharification. Means and standard errors are indicated.

Table 1: Enzyme activities from old and new (fresh) SG2 culture

Treatment	Cellulase	Xylanase	β-glucosidase	β-Xylosidase
SG 2 Old	4.53	38.67	23.15	4.42
SG2 Fresh	5.37	39.66	19.45	4.15

Table 2 Compositional analyses of feedstock samples used in this project

W/W%	Crude protein	Crude fiber	ADF	NDF	Ash	Cellulose	Lignin	Hemicellulose	Reducing Sugars
Switchgrass 1	6.31	33.95	40.52	78.30	4.76	33.89	5.75	37.78	3.07
Acid-treated switchgrass 1	5.30	48.90	64.96	76.06	2.44	53.12	10.15	11.10	0.47
Switchgrass 2	2.55	43.31	51.69	83.53	1.48	41.59	9.97	31.84	1.42
Acid-treated switchgrass 2	1.89	60.00	82.30	84.63	0.52	59.34	22.87	2.33	0.40
Gamma grass	5.64	35.41	46.97	74.94	3.05	34.53	11.29	27.98	1.03
Acid-treated gamma grass	5.49	48.30	73.97	76.15	1.02	57.36	16.16	2.19	0.15
Saw dust	2.46	48.86	62.62	72.46	1.58	46.49	16.16	9.84	0.36
Acid-treated saw dust	2.09	58.56	82.56	82.22	0.63	57.08	25.60	0.00	0.05

Switchgrass 1 was collected from Union Springs, AL. Switchgrass 2 and Gamma grass were collected from Auburn, AL.

Conclusions:

Effect of switchgrass particle size on saccharification by crude *Trichoderma* SG2 enzyme

- The effect of switchgrass particle size on saccharification could not be conclusively determined for particles 1mm and lesser. All the experiments conducted thus far have used 2mm switchgrass and the data from xylose yield showed that 2mm is the best followed by 3mm and 4mm particle size. In case of glucose, 3mm was found to be the best. So it can be inferred that 2 to 3mm particle size is ideally suited for saccharification.
- Baig et. al. (2004) reported that in banana plant waste the greater sugar yield was reported with reduced particle size. However in our present study the best yield was obtained with particle size between 2-3mm.

Effect of switchgrass feedstock concentration on saccharification by crude *Trichoderma* SG2 enzyme

- Of the five different feedstock concentrations (2%, 5%, 10%, 15% and 20%) tested, 5% yielded the highest sugars upon saccharification with SG2 crude enzyme. Although 10% showed the overall highest sugar yield, it was due to very high yield at 72h. The corresponding yields at 24h and 48h were lesser than that for 2% and 5%. So

the yield from 10% concentration needs validation. Higher concentrations of 15% and 20% make the medium too thick which does not allow mixing and the final product has little or no liquid for sampling and hence the huge variation in Fig 2a and b. Presence of liquid is vital for proper enzyme action on the feedstock. So using high substrate concentrations is ruled out. Feedstock concentrations of 2% or 5% seem to be suited well for saccharification. Although 5% gives the highest sugar yield, the yield is not proportionately increasing when compared with 2%.

Effect of SG2 saccharification of paper powder

- Acid-pretreated paper powder at 5% concentration yielded 10g/L glucose after 48h. Accordingly, white-paper waste generated can be utilized for saccharification and potential biofuel production. It could be used to supplement switchgrass feedstock.
- White paper with and without print was used in this experiment. Untreated paper yielded negligible sugar whereas acid pretreatment was able to yield substantial sugars with SG2 crude enzyme.

Effect of paper powder supplementation to switchgrass feedstock on sugar yield by crude SG2 enzyme

- **Supplementation** of paper powder to switchgrass feedstock at 50:50 to get a total of 6% resulted in high glucose and xylose levels (Figs. 3a, b) which was slightly greater than that obtained at 5% switchgrass alone (Figs. 2a, b).
-

Repeated enzyme addition to saccharified switchgrass to obtain maximum sugar yield

- The sugars obtained by repeated saccharification of saccharified-switchgrass did not result in high sugar levels. The data obtained when compared with regular saccharification for 72h (Fig. 2a, b) shows the following:
  - **Glucose** from **2% switchgrass** after 72h regular saccharification is **9.4g/L** whereas cumulative yield after repeated extraction is **7.5g/L**
  - Glucose from **5% switchgrass** after 72h regular saccharification is **11.7g/L** whereas the cumulative yield after repeated saccharification is **13.3g/L**
  - **Xylose** yield from **2% switchgrass** after 72h regular saccharification is **2.4g/L** whereas the cumulative yield after repeated extraction is **2.3g/L**
  - Xylose yield from 5% switchgrass after 72h regular saccharification is **2.7g/L** whereas the cumulative yield after repeated extraction is **4.5g/L**
- Repeated saccharification of saccharified-biomass does not enhance overall sugar yield as compared with one-time enzyme addition at least in the case of acid-pretreated switchgrass.
- Using 10ml enzyme in one-time addition of crude enzyme to switchgrass yields far greater sugar when compared with 30ml enzyme addition by way of repeated saccharification. Even if there were marginal increases in sugar yield (as seen in glucose and xylose yield from 5% switchgrass), it is not proportionate to the amount of enzyme used.

Effect of enzyme age on switchgrass saccharification

- In the last quarter, activities of enzyme from fresh and three month old *Trichoderma* SG2 were studied and it was found that the activities remained unchanged. In this quarter our data shows that the sugar yields due to saccharification from enzymes from fresh and two-month old culture were not different irrespective of the feedstock concentration used. It can be concluded that *Trichoderma* SG2 culture age does not affect enzyme activities or saccharification.

Scale up validation of saccharification and ethanol fermentation of switchgrass by *Trichoderma* SG2

- The maximum glucose yield after 72h was 6.4g/L and xylose was 1.7g/L by SG2 crude using 5% switchgrass. In the last quarter, the maximum glucose yield of 4.97g/L and xylose yield of 1.37 g/L respectively were obtained from 2% switchgrass after 72h saccharification with C50N.
- This study yielded 12.8% of glucose which was produced from 60% of cellulose (based on compositional analysis; see Table 1) indicating 21% conversion of cellulose to glucose.
- About 2.84g of ethanol was released from 6.4g glucose. The theoretical yield should have been 3.37g. Therefore **87% conversion efficiency** of ethanol was seen glucose from **5% switchgrass** saccharification. In the last quarter, 80% conversion efficiency of ethanol was seen.
- After saccharification and ethanol fermentation, inhibitors like acetic acid and furfural were not detected by HPLC and our detection limit was 10μg/L.
- Using 5% switchgrass may be more suited to obtain higher sugar and ethanol yield with higher ethanol conversion efficiency.
- Saha et al (2005) reported in a simultaneous saccharification and fermentation of dilute acid pre-treated 1 % wheat straw the total ethanol yield was 0.24g/g of dry solids. However, in our study with crude enzyme in dilute acid-pretreated 2% switchgrass the yield of ethanol after 24h is 0.06g/g of dry solids. Their study of course used commercial recommended doses.

Milestones outlined for tasks A, B and C have been accomplished as outlined in the previous report. A 7L fermenter has been procured and process optimization of large-scale enzyme production, saccharification and ethanol fermentation will be carried out to optimize the conditions to reach the milestone outlined in Task D. From this quarter we conclude that 5% switchgrass can be used to achieve greater sugar and ethanol yield.

Although we achieved the milestones outlined for tasks A, B and C in the last quarter, some parameters that may aid in process optimization like particle size of feedstock, supplementation of paper powder, substrate concentration, and effect of enzyme from aged culture on saccharification were studied. Further, to extract the maximum sugars possible from the feedstock, repeated saccharification of saccharified-feedstock was conducted but the

amount of sugars released and the amount of enzyme used does not make the process economical. So, we have now narrowed down on all the factors for feedstock saccharification and ethanol fermentation for scaled up study in bioreactor system. Further optimization of factors using a 7L fermenter will contribute towards achieving scale up of the process.

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PART 8: Research Activities of FY-13 (October 1, 2012 to December 31, 2012)
Written by: Benedict Okeke and Ananda Nanjundaswamy,

Introduction:
In the previous quarter, it was reported that *Trichoderma* SG2 is a stable culture for enzyme production and switchgrass saccharification, 2% acid-pretreated switchgrass has higher cellulose conversion to glucose efficiency than 5% substrate, different particle sizes of switchgrass subjected to pretreatment had no conceivable trend as far as sugar yield was concerned, paper waste generated in the form of 5% acid-pretreated paper powder can be a potential biofuel feedstock or supplement, supplementation of paper powder to switchgrass feedstock at 50:50 to get a total of 6% resulted in high glucose and xylose levels (which was slightly greater than that obtained at 5% switchgrass alone), single step saccharification was more efficient than multiple or repeated saccharification of biomass, and scale-up of 5% switchgrass saccharification and ethanol fermentation by SG2 crude enzyme resulted in 21% cellulose conversion to glucose and 87% ethanol conversion efficiency respectively. In this quarter the following objectives were addressed:

Specific Objectives
1. Effect of metal ions on the production of cellulolytic and xylanolytic enzymes from *Trichoderma* SG2 and saccharification of cellulosic feed stock.
2. Use of sand filtration as an effective tool for separation of fungal biomass from *Trichoderma* SG2 crude enzyme for the farm deployable bioreactor process.
3. Effect of tangential flow filtration (TFF) for concentration of *Trichoderma* SG2 crude enzyme and its effect on feedstock saccharification.
5. Scale-up bioprocessing reactor design and development.

Materials Methods

Effect of metal ions on the production of cellulolytic and xylanolytic enzymes from *Trichoderma* SG2 and saccharification of cellulosic feedstock

Nine different metal ions were examined by using their respective salts. Copper ($CuCl_2$), Barium ($BaCl_2$), Iron ($FeCl_2$), Potassium (KCl), Calcium (CaCl), Magnesium ($MgCl_2$), Zinc ($ZnCl_2$), Nickel ($NiCl_2$), Cobalt ($CoCl_2$) and Ethylenediaminetetraacetic acid (EDTA) a polyaminocarboxylic acid that chelates metal ions were used. All chemicals of analytical grade were obtained from VWR.

Media preparation:
Crude enzyme production was carried out in 250ml flasks containing 100ml optimized screening medium with or without the respective metal ions. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80 and 10mM of respective ion. One liter control medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH2PO4, 1.2g (NH4)2SO4, 0.5g MgSO4.7H2O, 0.1g CaCl2, 0.003g FeSO4.7H2O, and 2ml of Fotch mineral element solution. pH varied from 5.2 to 5.35 and did not warrant any further modification). Flasks were sterilized at 121deg C for 60 min and cooled to room temperature.

Seed inoculum Preparation:
About a loop full of SG-2 culture was inoculated into 50ml sterile high-solids medium in a 250ml flask containing 5% all-purpose flour, 0.1% yeas extract and 0.1% peptone. Flasks were incubated at 30°C for 72 at 200rpm.

Enzyme production:
About 10% of 72h inoculum was aseptically added into each 250ml flask containing 50ml of screening medium with or without ions, incubated at 30°C for 5 days. Each treatment received three replicates. On 5^{th} day of enzyme production, the broth was transferred into sterile 50ml centrifuge tubes and centrifuged at 5000rpm for 10 min. The supernatant was used for evaluation of enzyme activities (cellulase, xylanase, beta-glucosidase and beta-xylosidase) and biomass saccharification.

Enzyme assays
End of fermentation samples were analyzed for cellulase, xylanase, β-glucosidase and β-xylosidase. Method described in Saha et al (2005) as modified by Okeke and Lu (2011) was followed. Briefly, filter paper discs were used for cellulase assay. About 10 discs were placed in glass test tubes and 0.5ml of 100mM sodium acetate buffer at pH 5.0 was added. About 0.5ml of end of fermentation sample (crude enzyme extract) was added and tubes were incubated at 50ºC for 30min. Enzyme reaction was stopped with DNS reagent and 40% sodium potassium tartarate, sample mixture placed in a boiling water bath for 10min and cooled to room temperature. Using a spectrophotometer, optical density was read at 575nm, and mg/ml of reducing sugar released was calculated using standard glucose curve. The same procedure was followed for xylanase assay except that the substrate was oats xylan.

For β-glucosidase and β-xylosidase, substrates *p*-nitrophenyl β-D-glucoside and *p*-nitrophenyl β-D-xyloside were respectively used. In brief, approximately 800µl of 100mM acetate buffer was added to 100ml of 40mM of substrate. About 100µl of crude enzyme extract was added, incubated at 50ºC for 30 min and immediately transferred onto ice cold water bath. The enzyme reaction was stopped using 1ml of chilled Na_2CO_3. Sample ODs were read at 405nm and enzyme activity expressed as micro moles of p-nitrophenol released per milliliter of enzyme extract was calculated by using standard curve.

Feedstock Pretreatment
Acid-pretreatment of switchgrass was carried out using 2% sulfuric acid. About 100g of switchgrass was soaked in 2% sulfuric acid and autoclaved at 121° C for 1h. After autoclaving the contents were allowed to cool to room temperature, liquid drained using a cheese cloth and the acid-pretreated switchgrass washed several times with water to completely remove the acid residues. After 4-5 washes the pH of the liquid was tested and was washed further if the pH was less than 5. The washed switchgrass was dried at room temperature for 72h and stored in airtight ziplock bags until further use.

Saccharification
Saccharification was carried out in 100ml flasks with airtight caps. About 10ml of reaction mixture containing 5% acid-pretreated switchgrass was taken in each flask. All experiments were carried out in triplicates. Saccharification was carried out at 50°C at 80rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All the flasks were closed tightly to prevent loss of moisture. About 0.5ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Effect of sand filtration as an effective tool for separation of fungal biomass from Trichoderma SG2 crude enzyme

Crude enzyme production was from *Trichoderma* SG2 carried out in ten 250ml flasks containing 100ml optimized screening medium. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution. pH varied from 5.2 to 5.35 and did not warrant any further modification). Flasks were sterilized at 121°C for 1h before inoculation. About 10% of 72h inoculum was added into each flask aseptically, incubated at 30°C for 5 days. On 5th day of enzyme production, broth from 10 flasks were pooled and stored at 4°C to protect enzyme activity during filtration. From one liter broth, 300ml was used for sand filtration, 100ml for centrifugation, a small portion was used for enzyme assays and the remainder stored at 4°C. For centrifugation which served as a control, about 100ml of broth was transferred into sterile 50ml centrifuge tubes and centrifuged at 5000rpm for 10 min.

Sand filter:

Sand column (1.5cm diameter, 15cm length) for filtration was prepared by using about 100g of 40-100 mesh sized acid-washed sand was loaded into transparent polycarbonate columns. The experiment involved three replicates). About 100ml of the cold fungal broth containing enzyme was loaded onto the column and filtrate was collected in a sterile centrifuge tube placed in an ice bath. This experiment was repeated thrice. Collected sample was used for enzyme assay and for saccharification. As a control, part of the sample was centrifuged at 5000 rpm for 15min and was used for assay as well as saccharification. Enzyme assays and saccharification of 5% acid-pretreated switchgrass was carried out as detailed earlier. For saccharification, two replicates were maintained for each treatment.

Use of tangential flow filtration (TFF) for concentration of *Trichoderma* SG2 crude enzyme and its effect on feedstock saccharification

Enzyme production

Enzymes were produced using the medium described earlier. Enzyme samples were centrifuged at 5000rpm for 15min and pooled carefully to avoid any physical particles.

TFF setup:

Millipore Pelicon Labscale TFF system fitted with Pelicon cassettes (Biomax Polyethersulfone membranes with 5000 Da molecular weight cut off membrane size) was used for concentrating the centrifuged crude enzymes. TFF set up and operations were carried out as per the instruction provided in the Pelicon Labscale TFF manual and briefly described below. Samples were concentrated two, five and ten-folds. Both permeate and retentate (concentrated enzyme) were collected in separate containers and stored in refrigerator to prevent enzyme activity losses.

In brief, about 100 ml of centrifuged crude enzyme was taken in the 500 ml reservoir and constantly mixed using the magnetic stirrer and pumped through Pelicon cassette. Pressure was monitored to ensure it did not exceed 20psi.

Following steps as described in the manual were followed for concentrating enzyme by TFF:

1. Reservoir was filled with enzyme sample.

2. Vent port was unplugged, installed with a Millex Filter and tank outlet valve left open.
3. Pump was turned on until feed pressure gauge read 1.38 Bar (20psi). System connections for leaks were checked and tightened if needed.
4. Retentate valve restriction was adjusted to read 0.69 Bar (10psi) by slowly turning the retentate valve clockwise.
5. Pump speed and retentate valve restriction was adjusted to achieve desired feed retentate pressures (recommend 2.07 Bar (30psi) feed / 0.69 Bar (10psi) retentate).
6. Pump outlet tubing was disconnected (Sta-Pure, white) from pump outlet port and placed in product recovery collection vessel.
7. Retentate tubing (silicone, translucent) was disconnected and fluid drained by gravity. To remove additional drainage, a syringe was placed on the end of the retentate tube and fluid blown down.

Concentrated samples were used for enzyme assays and saccharification as outlined above, and total protein estimation was conducted using Bradford's method (Bradford 1976).

Statistical analyses

When required data was statistically analyzed using Statistical Analysis Software (SAS version 9.2). *t-test* was conducted and pair-wise differences were indicated when treatments did not share a letter among them. Significance was set at P=0.05.

Results and Discussion

Effect of metal ions on the production of cellulolytic and xylanolytic enzymes from *Trichoderma* SG2 and saccharification of cellulosic feed stock

The enzyme activities of crude enzyme by *Trichoderma* SG2 when grown on media amended with different metal ions is presented in Fig.1 and the saccharification of acid-pretreated switchgrass from respective enzyme samples is presented in Fig. 2. *t-test* shows that there were significant differences among treatments as indicated by pair-wise differences (treatment not sharing a letter are significantly different in the following):

Cellulase (P<0.0001) $Mn^{a}>Control^{b}>Ba^{c}>K^{dc}>Zn^{dc}>Mg^{dc}>Ca^{d}>Fe^{e}>EDTA^{e}>Co^{f}>Cu^{f}>Ni^{f}$

Xylanase (P<0.0001)

$Mn^{a}>Control^{b}>K^{cb}>Ca^{bcd}>Co^{cd}>Zn^{ced}>Ba^{ed}>EDTA^{ef}>Fe^{f}>Mg^{f}>Ni^{g}>Cu^{g}$

B-Glucosidase (P<0.0001)

$Mg^{a}>Zn^{b}>Ba^{bc}>Control^{bc}>K^{bc}>Mn^{c}>Ca^{d}>Fe^{e}>EDTA^{e}>Ni^{f}>Co^{f}>Cu^{f}$

B-Xylosidase (P<0.0001) $Mg^{a}>Ca^{b}>K^{b}>Ba^{c}>Control^{c}>Zn^{c}>Mn^{c}>Fe^{d}>EDTA^{e}>Co^{e}>Ni^{e}>Cu^{e}$

Glucose yield (P<0.0001) $K^{a}>Control^{a}>Mg^{a}>Ba^{b}>EDTA^{c}>Mn^{c}>Zn^{d}>Ca^{d}>Ni^{e}>Cu^{e}>Co^{e}>Fe^{e}$

Xylose yield (P<0.0001)

$K^{a}>Ba^{ab}>Control^{ab}>Mg^{ab}>EDTA^{bc}>Zn^{cd}>Mn^{d}>Ca^{d}>Co^{e}>Fe^{e}>Ni^{e}>Cu^{e}$

The highest cellulase and xylanase activities were found in medium amended with only Mn which was significantly greater than the control screening medium that had a suite of metal ions. In case of beta-glucosidase and beta-xylosidase activities, crude enzyme of SG2 grown on media amended with only Mg was significantly greater than the control. Interestingly, these enzymes with highest activities were not the ones that yielded the highest sugar yield: SG2 crude enzyme grown on media amended with only potassium resulted in the highest sugar yields, but were not significantly greater than control which implies that screening medium with a suite of metal ions and trace elements previously optimized can be used for saccharification of biomass.

Use of sand filtration as an effective tool for separation of fungal biomass from *Trichoderma* SG2 crude enzyme

The sand filtration set up and the product obtained by this process is outlined in Fig. 3. The enzyme activities of centrifuged SG2 crude enzyme sample (CT) and that of sand-filtered (SF) samples were not different from each other for cellulase (SF>CT; *P=0.7626*) and beta-glucosidase (CT>SF; *P=0.073*) whereas sand-filtered had higher xylanase activity than centrifuged sample (SF>CT; *P<0.0001*) and centrifuged sample had significantly higher beta-xylosidase activity than sand-filtered sample (CT>SF; *P=0.0301*) as shown in Fig. 4a. As for the sugar yields from saccharification of acid-pretreated switchgrass, the glucose yield was not significantly different between sand-filtered and centrifuged samples (CT>SF; *P=0.2073*) but for xylose yield, centrifuged sample yielded higher amount of sugar than sand-filtered samples (CT>SF; *P=0.0012*) as shown in Fig. 4b. The protein content between centrifuged and sand-filtered samples were significantly different (*P<0.0001*) with 0.83mg/ml protein in centrifuged sample and 0.63mg/ml in sand-filtered sample indicating 24% reduction in the protein content in sand-filtered samples (Fig. 4c).

Compared to CT sample, there was 9% and 48% reduction in glucose and xylose yield respectively. Both cellulase and xylanase activities in SF increased by 1% and 17% respectively, whereas the beta-glucosidase and beta-xylosidase activities were reduced by 5% and 9% in SF compared to CT.

Despite the reduction in protein content, the glucose yield was unaffected but the xylose yield was reduced. Overall, sand filtration can easily substitute centrifugation to separate fungal biomass from SG2 crude enzyme.

Effect of tangential flow filtration (TFF) for concentration of *Trichoderma* SG2 crude enzyme and its effect on feedstock saccharification

The enzyme activities of SG2 concentrated by TFF by two-fold (2F), five-fold (5F) and ten-fold (10F) are outlined in Fig. 5. The activities in crude, retentate (concentrated enzyme) and permeate (residue lacking in enzyme) were compared. As expected for all enzymes except xylanase, permeate hardly contained any enzyme (Fig. 5a-c). To draw meaningful conclusions, only activities of crude and retentate were compared statistically:

Cellulase (*P<0.0001*) $10F^{a}>5F^{a}>2F^{b}>Crude^{b}$

Xylanase (*P=0.2596*) 2F>Crude>5F>10F

Beta glucosidase (*P=0.0019*) $5F^{a}>10F^{b}>2F^{b}>Crude^{b}$

Beta xylosidase (*P<0.0001*) $5F^{a}>10F^{b}>Crude^{c}>2F^{d}$

Overall five-fold concentration was the best in retaining high enzyme activities followed by ten-fold concentration. Obviously, tangential flow filtration concentrated the enzyme and the activities were all greater than that of crude enzyme. TFF seems to have absolutely no influence on xylanase enzyme: both retentate and permeate contained enzymes when in fact permeate should have had no enzyme at all. The enzyme surely evaded the concentration process possibly due to the small molecular size of xylanase.

Both, crude enzyme and retentate from TFF were used for saccharification of acid-pretreated switchgrass (Fig. 6a). As far as sugar yield from saccharification of acid-pretreated switchgrass was concerned:

Glucose and xylose (*P<0.0001*) $10F^{a}>5F^{b}>Crude^{c}>2F^{d}$

Ten-fold concentration of enzyme resulted in highest sugar yield followed by five-fold enzyme concentration.

The effect of TFF enzyme concentration on enzyme concentration was determined by quantifying the protein content in the samples (Fig. 6b). The protein content between crude and retentate samples was also compared:
Protein content (*P<0.0001*) $10F^{a}>5F^{b}>2F^{c}>Crude^{d}$
The highest protein content was seen in ten-fold concentration followed by five-fold concentration.

Specific activity is the enzyme activity per mg of protein which is more accurate representation of enzyme activity. The specific activities of crude and retentates were also compared (Fig. 6c):
Cellulase (P<0.0001) $Crude^{a}>2F^{b}>5F^{c}>10F^{d}$
Xylanase (P=0.0004) $Crude^{a}>2F^{a}>5F^{b}>10F^{c}$
Beta glucosidase (P=0.0018) $5F^{a}>2F^{a}>Crude^{a}>10F^{b}$
Beta xylosidase (P<0.0001) $5F^{a}>$ $Crude^{b}>2F^{c}>$ $10F^{c}$

Overall, TFF was able to concentrate enzyme and the ten-fold concentration was the best as far saccharification and protein content were concerned.

Table 1 compares the overall enzyme activity, protein content and specific activity of commercial and in-house crude enzymes.

Scale-up bioprocessing reactor design and development
A scale-up reactor vessel has been designed to specifically meet the needs of this project. The 1350L (357 US gallons) bioprocessing reactor with a working volume of 1000L (264 US gallons) is useful for biomass pretreatment, enzyme fermentation, feedstock saccharification and ethanol fermentation and initial dilute ethanol recovery. The double-jacketed stainless steel vessel is envisioned to have an outer jacket (304 grade) and inner tank (316L grade) to withstand atmospheric pressure and 0.2MPa respectively. The double-jacket will contain heating coils along with heating oil with a total heating area of $3.8m^2$. There will be at least two oil inlets and one oil outlet ports. The vessel will have five inlets for fed-batch additions. Further, the vessel will have the usual features like cleaning hole, breath hole, manhole with sight glass and sight light, pressure hole, air pump relief valve, sample hole and outlet will be fitted. Two Rushton impellors will be connected to 1.5kw motor with 0-500rpm capacity. The vessel will be fitted with wheels for easy movement of the vessel. We have procured a blue-print of a custom-made bioreactor (Fig. 7) from Zhejiang Dayu Light Industrial Machinery Co. Ltd., (Wenzhou, Zhejiang, Peoples Republic of China). This vessel is substantially less expensive than other vessels we have considered to procure for the bioprocess. Considering the working volume of the vessel and our data (July 2012) concerning the effect of reaction volume on enzyme production, the vessel can easily process 10kg of acid-pretreated switchgrass.

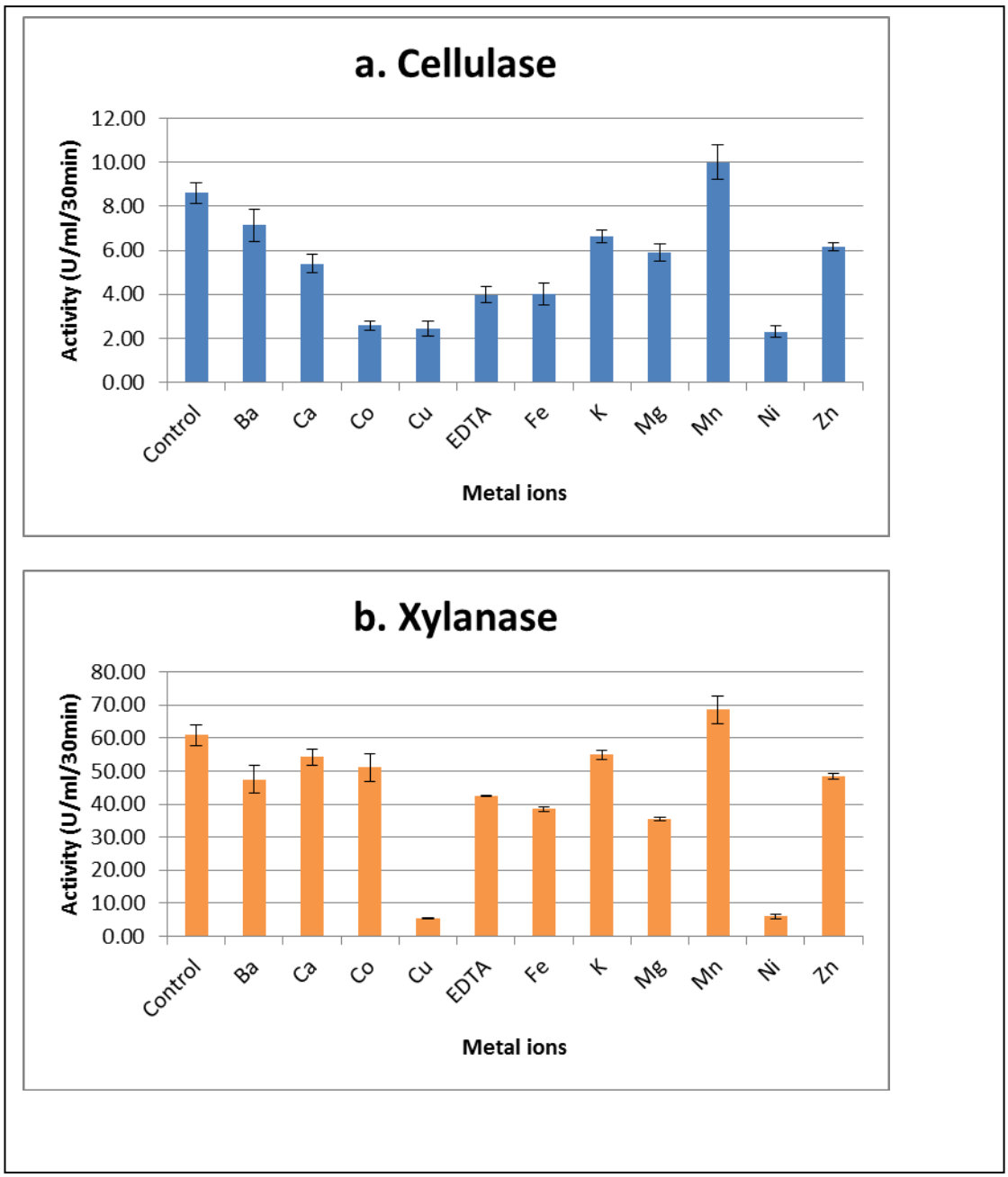
Table 1: Enzyme activities of 'Commercial and In-house *Trichoderma* SG2 crude enzyme

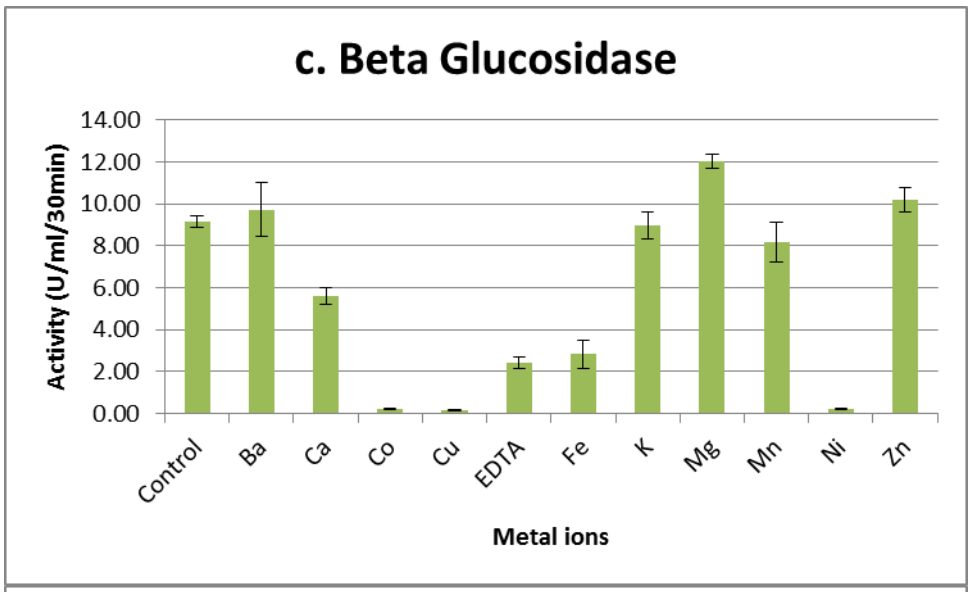
Enzymes	Activity (U/ml/30min)	Protein (mg/ml)	Specific Activity (u/mg)
In-house crude -Cellulase	10.93 ±0.07	0.82±0.00	13.33±0.08
In-house crude-xylanase	44.33±0.00	0.82±0.00	54.06±0.4
In-house crude-β-glucosidase	17.88±0.00	0.82±0.00	21.80±0.00
In-house crude-β-xylosidase	06.22± 0.09	0.82±0.00	7.59±0.10
Commercial Cellulase	31.46± 0.14	54.8± 0.01	0.57±0.00
Commercial Xylanase	55.21± 0.22	58.4 ±0.01	0.95±0.00
Commercial Beta-Glucosidase	24.72 ±0.09	58.6±0.009	0.42±0.00

Trichoderma SG2 crude enzyme was the non-concentrated culture filtrate.

Figures

Figure 1





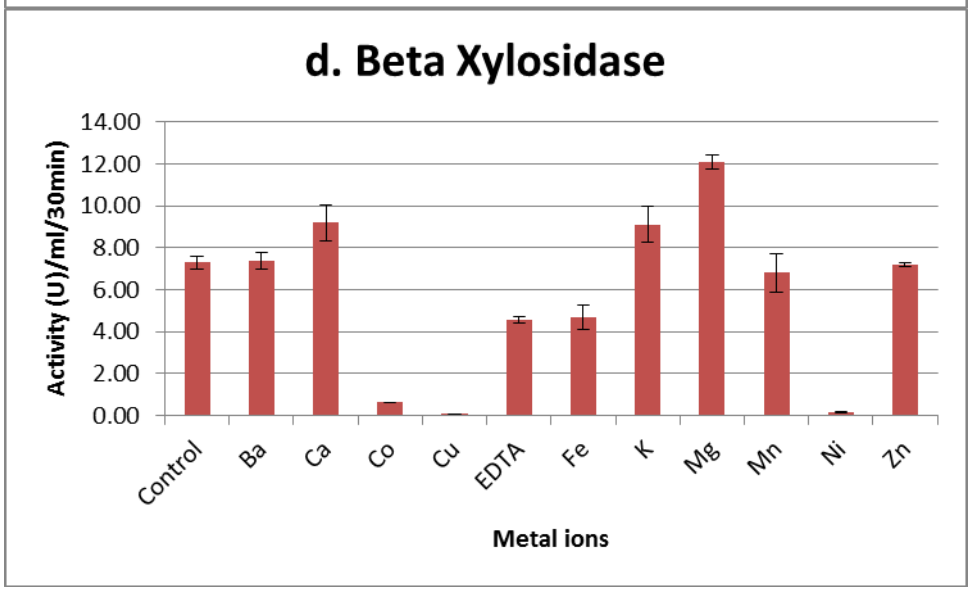


Figure 38 Enzyme activties of Trichoderma SG2 enzyme produced on media amended with metal ions. a. Cellulase b. Xylanase c. Beta Glucosidase d. Beta Xylosidase. Means and standard errors are indicated. Pair-wise differences indicated in text.

Figure 2

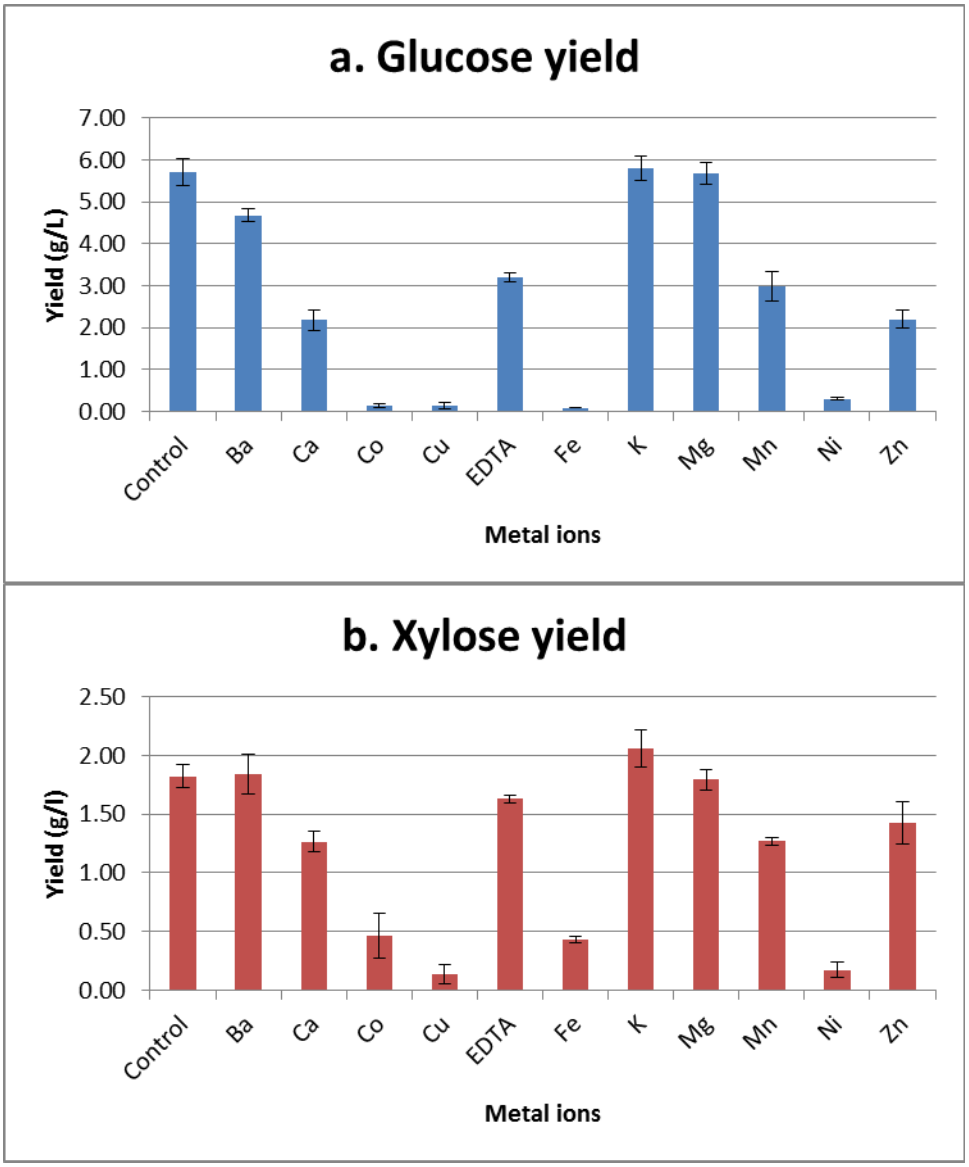


Figure 39 Saccharification of 2% acid-pretreated switchgrass by Trichoderma SG2 enzymes produced from media amended with metal ions. a. Glucose yield b. Xylose yield. Means and standard errors are indicated. Pair-wise differences are resented in text.

Figure 3

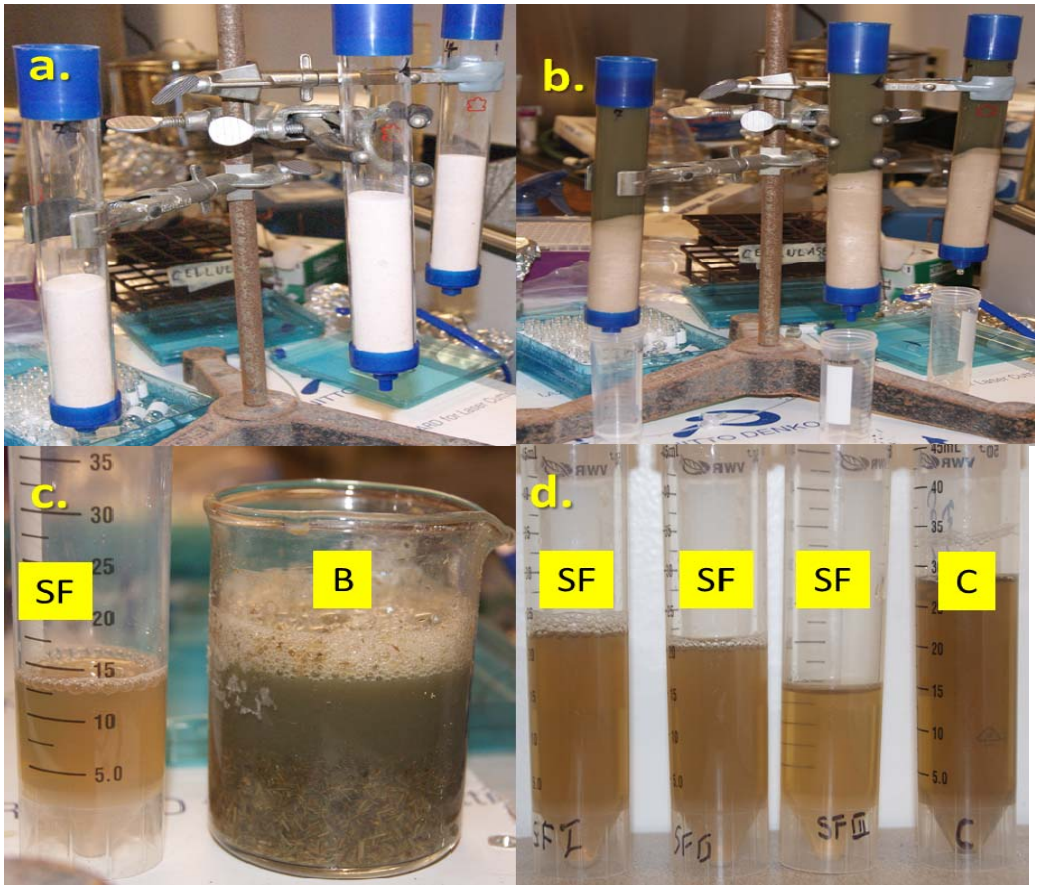
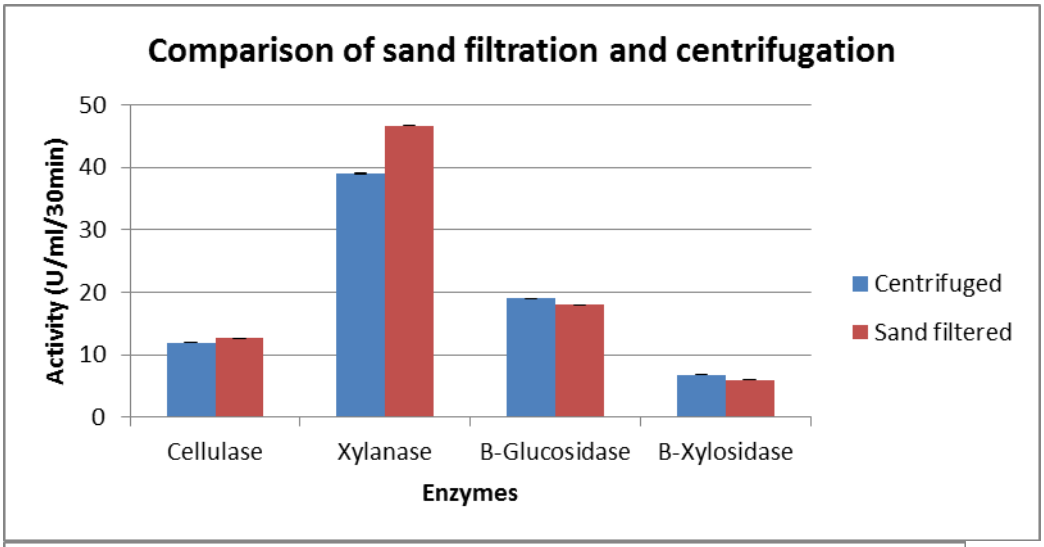
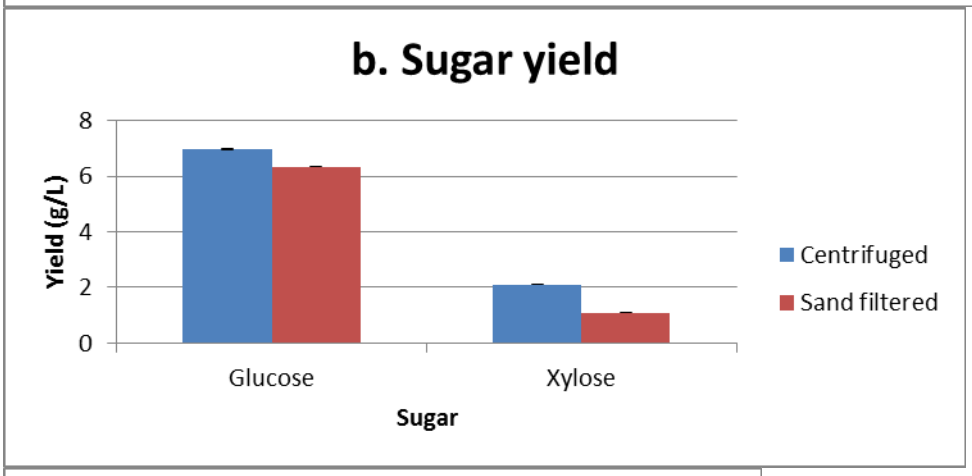


Figure 40 Sand filtration a. set up of sand filters b. sand filters loaded with SG2 crude enzyme c. sand filtered sample (SF) compared to original crude enzyme broth (b) d. three sand filtered (SF) samples compared to centrifuged (C) sample

Figure 4





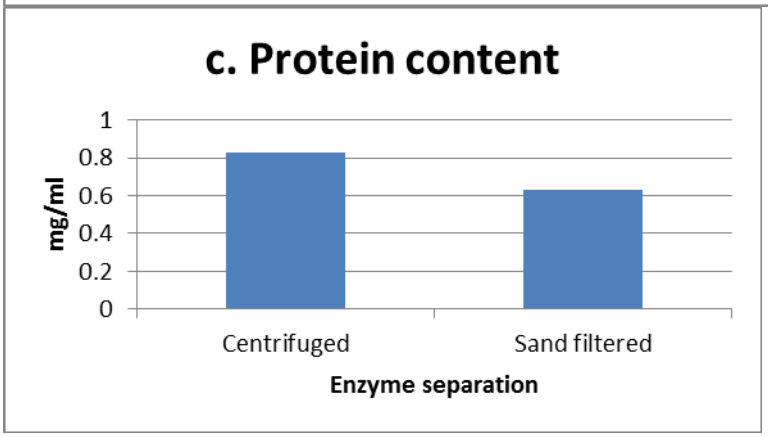
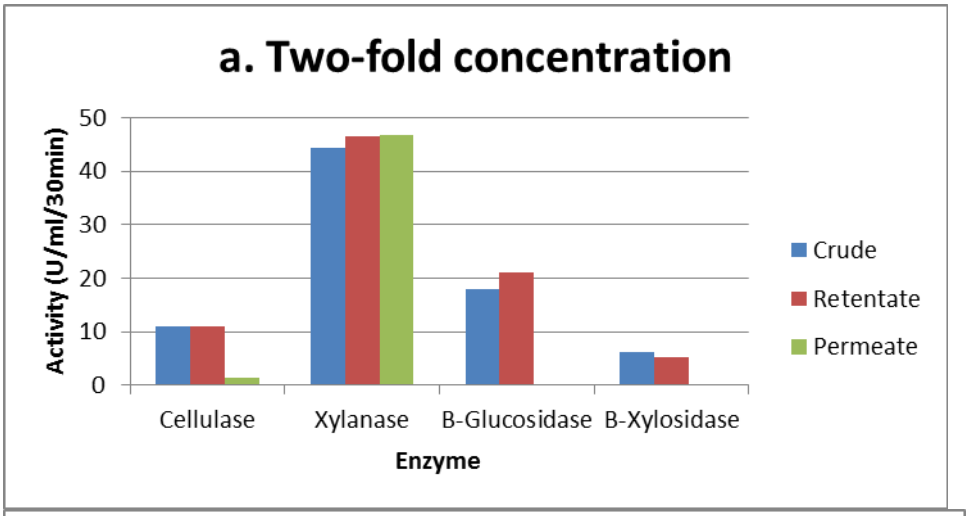
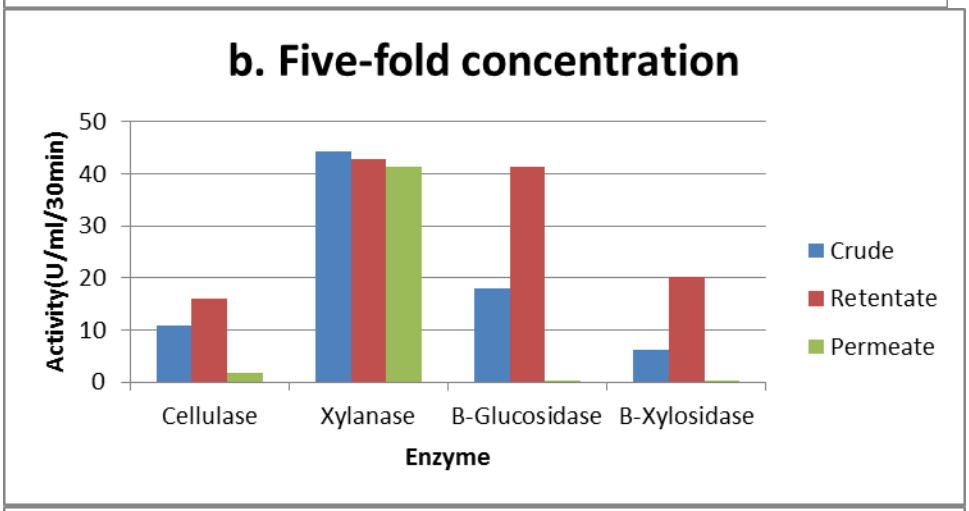


Figure 41 Comparison of sand-filtered and centrifuged crude enzyme from Trichoderma SG2. Means and Standard errors are indicated. a. Enzyme activities b. Saccharification c. Protein content.

Figure 5





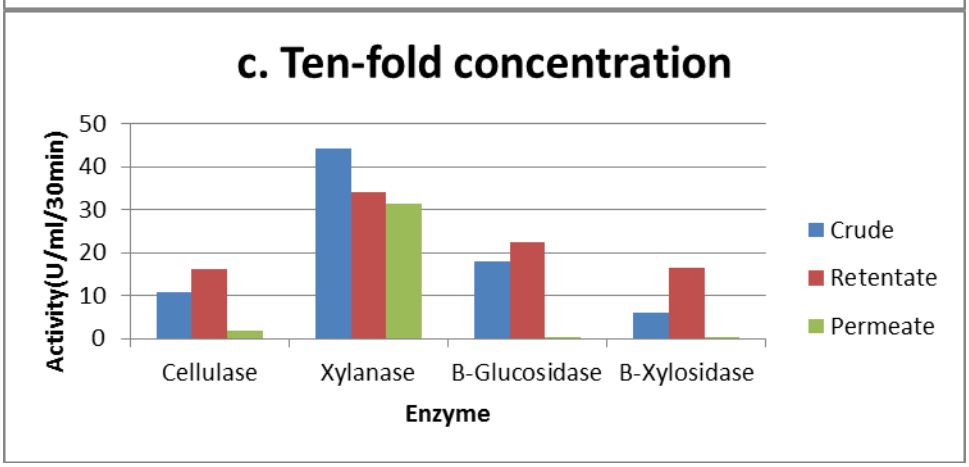


Figure 42 Enzyme activities from SG2 crude enzyme upon concentration using tangential flow filtration a. Two-fold b. Five-fold c. Ten-fold. Means are indicated. Pair-wise differences are indicated in the text.

Figure 6

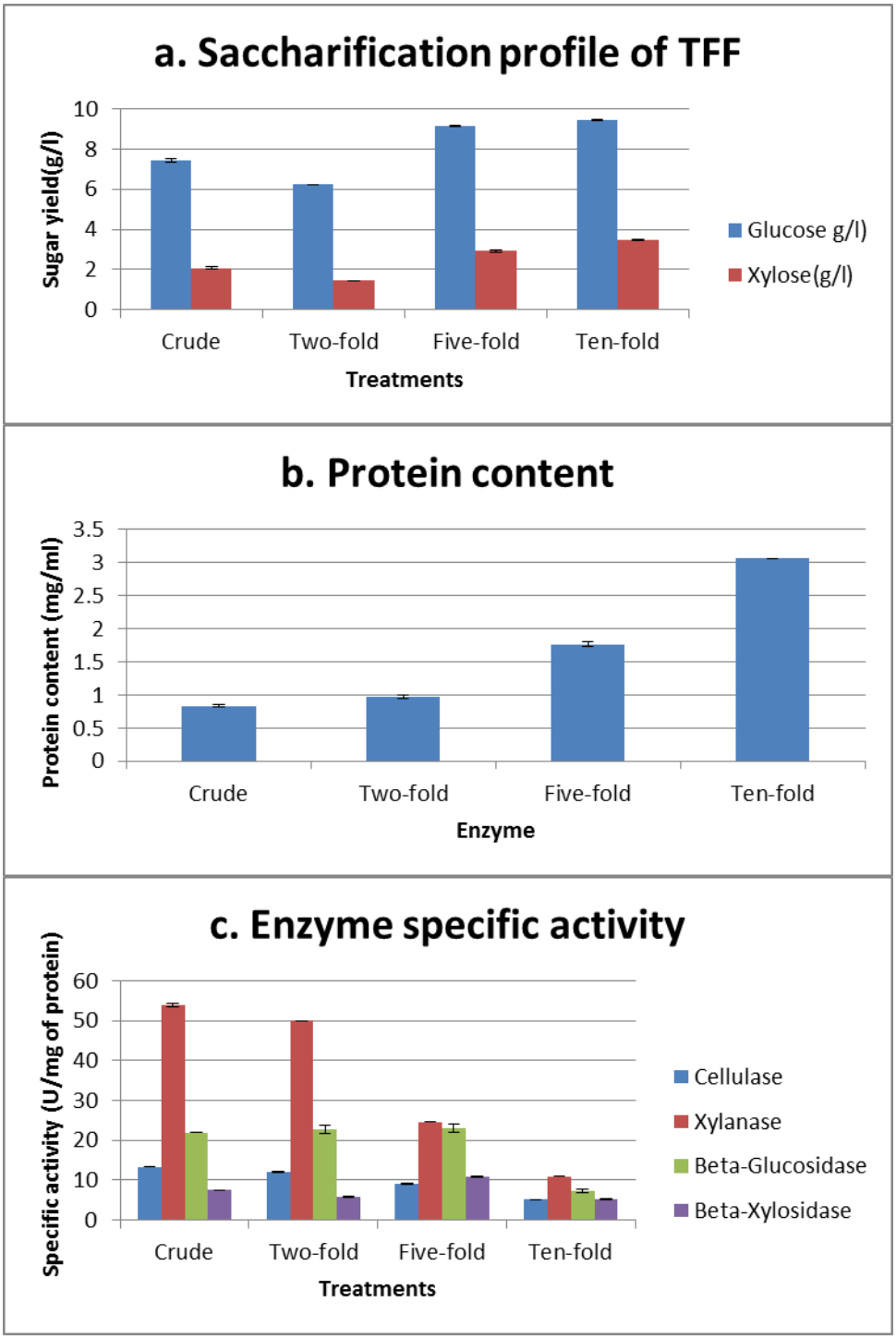


Figure 43 Comparison of crude enzyme and retentate samples from TFF. a. Saccharification b. Protein content. c. Specific activity. Means and standard errors are indicated. Pair-wise differences are indicated in the text.

Figure 7

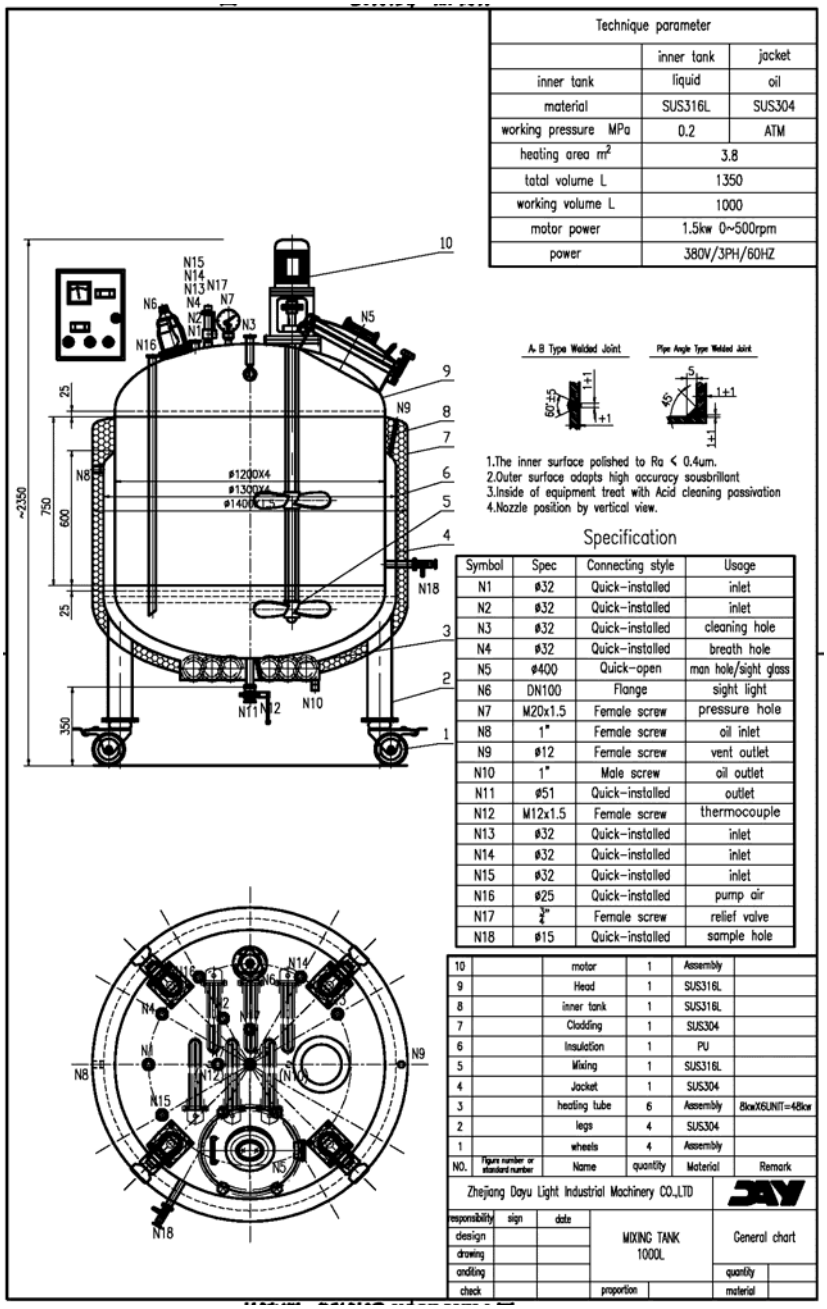


Figure 44 Blue-print of custom-made bioprocess vessel on wheels for scale-up. This is a 1350L capacity bioreactor vessel capable of utilizing 10kg switchgrass and is being considered for this process. Alternate vessel from Allard Research is also being considered.

Conclusions and Milestones:

Effect of metal ions on the production of cellulolytic and xylanolytic enzymes from *Trichoderma* SG2 and saccharification of cellulosic feed stock

- The cellulase and xylanase activities were the highest when the growth medium contained 10mM of only Manganese followed by the screening medium (control) containing a suite of metal ions which resulted in significantly lesser activities. The beta-glucosidase and xylosidase activities were highest when Magnesium was used as the sole metal ion and their respective activities in the control were nowhere close to that of Mg.
- The growth media for enzyme production were amended with 10mM Mg or Mn whereas the screening medium (control) contained 0.2mM of Mg and 0.325mM of Mn.
- In contrast, Damisa et al (2008) in their study with *Aspergillus niger* for cellulase production reported that different concentrations of manganese did not increase the cellulase activity.
- Interestingly, the highest sugar yields from saccharification did not result from enzymes produced from media with Manganese or Magnesium but from that with potassium amendment, which was not significantly different from that of the control.
- Overall, further amendment of our optimized production medium with metal ions may not be necessary for enzyme production considering that it is a complex growth medium containing paper powder, switchgrass, peptone and yeast extract as well as a suite of trace metal ions that could provide most nutrients including trace minerals required for *Trichoderma* SG2 growth.

Use of sand filtration as an effective tool for separation of fungal biomass from Trichoderma SG2 crude enzyme

- The activities of cellulase and beta-glucosidase both yielding glucose from lignocellulosic feedstock namely switchgrass were unaffected by sand-filtration compared to centrifugation whereas activities of enzymes responsible for xylose release namely, xylanase and beta-xylosidase from sand filtration were either greater or lesser than centrifuged samples respectively.
- This was reflected in the subsequent saccharification of switchgrass where the glucose yields were similar between sand-filtered and centrifuged samples whereas the xylose yield in centrifuged sample was greater than that from sand-filtered sample.
- There was at least 24% loss in protein content in sand-filtered enzyme, yet this did not affect the cellulase and beta-glucosidase activities, resulting in glucose yields similar to that from centrifugation. There was 1% increase in cellulase activity, 5% reduction in beta-glucosidase activity and 9% reduction in glucose yield in sand-filtered sample compared to centrifuged sample, all of which are not reflected by the 24% loss in protein by sand filtration.
- Considering no significant loss of glucose yield by sand filtration (Fig. 4b), and the fact that a glucose-fermenting yeast namely *Saccharomyces cerevisiae* was employed for ethanol fermentation, sand filtration can be effectively employed to separate

Trichoderma SG2 biomass from crude enzyme and is an economical substitute to centrifugation.

- Interestingly, there was 17% increase in xylanase activity, 9% reduction in beta-xylosidase activity and 48% decrease in xylose yield in sand-filtered samples compared to centrifuged sample. Xylose yield is probably influenced by bioactive components lost or reduced during sand filtration and is probably reflected in the 24% loss of protein by sand filtration.
- Sand filtration has been used to effectively separate bacteria in river water by three-folds (Hendel et al 2001) and cryptosporidium oocysts from water (Chapman and Rush 1990). Additionally, Hendel et al (2001) reported reduction of some extracellular beta-glucosidase after sand filtration. We also noticed a marginal reduction in beta-glucosidase activity but the reduction was not statistically significant when compared to the centrifuged sample.

Effect of tangential flow filtration (TFF) for concentration of *Trichoderma* SG2 crude enzyme and its effect on feedstock saccharification

- Using TFF, the crude SG2 enzyme was concentrated two-, five- and ten-fold with concomitant increase in protein content, and sugar yield from saccharification of switchgrass. Ten-fold-concentrated enzyme was the best as far as sugar yield and protein content was concerned.
- However, it should be noted that although TFF increased protein content in the samples, there was substantial loss of protein based on mass balance: there was 40% loss, 58% and 56% loss of protein in the two-, five- and ten-fold concentrated enzyme samples.
- According to the Millipore manual (link appended in References) for the instrument, loss of protein from TFF is usually attributed to adsorption and/or solubility losses or incorrect operation of TFF and can be addressed by running the process multiple times or optimizing certain parameters.

Scale-up bioprocessing reactor design and development

- The custom-made 357gal ‘fermentor-on-wheels’ can easily process 10kg of acid-pretreated switchgrass and with our present ethanol production rate from one-liter fermentation using 2% switchgrass, we expect to produce at least one-liter of ethanol.

Presentations:

1. Nanjundaswamy A, Starr C, Okeke B. Processing of Biomass-Saccharifying Enzymes by Sand Filtration for 'Farm Deployable Microbial Bioreactor' Laboratory Model. ***American Society of Microbiology***, Denver, CO, May 18-21, 2013
2. Nanjundaswamy A and Benedict Okeke. Potential cost reduction of cellulosic biomass saccharification by fungal crude enzyme–commercial enzyme cocktail. ***CleanTech 2013***, Washington DC, May 12-15, 2013

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1. Ananda N, Vadlani PV, Vara Prasad PV (2011). Drought and heat stressed grain sorghum (*Sorghum bicolor*) does not affect the glucose and ethanol production. Industrial Crops and Products 33:779-782
2. Bradford, M.M. (1976), "Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Anal. Biochem. 72: 248–254
3. Chapman P. A. and Rush B. A. (1990) Efficiency of sand filtration for removing cryptosporidium oocysts from water. J. Med Microbiol 32243-245
4. Damisa D, Ameh J B, and Umoh V J (2008) The effect of changes in manganese concentrations on cellulase yield from bagasse fermented with mutagenised strain of *Aspergillus niger* AH3. Int. J. Biol. Chem. Sci. 2 (3) 368-372
5. Hendel B, Marxsen J, Fiebig D, Preuss G. (2001) Extracellular enzyme activities during slow sand filtration in a water recharge plant. Water Research 35(10) 2484-2488.
6. Millipore TFF manual: http://wolfson.huji.ac.il/purification/PDF/dialysis/MILLIPORE_TFF.pdf
7. Saha B C, Iten L B, Cotta M A and Wu Y V (2005) Dilute acid pre-treatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry 40 3693-3700.

PART 9: Research Activities of FY-13 (January 1, 2013 to March 31, 2013).
Written by: Ananda Nanjundaswamy and Benedict Okeke

Introduction:
In the previous quarter, it was reported that sand filtration was an inexpensive alternative to centrifugation to separate fungal mycelia from crude enzyme. Additionally, tangential flow filtration was successfully employed to concentrate SG2 crude enzyme two-, five- and ten-fold. Highest protein concentration was obviously seen in ten-fold concentrated sample which also resulted in highest sugar yield upon saccharification of 2% acid-pretreated switchgrass. In this quarter the following objectives were addressed:

Specific Objectives
1. Validation of switchgrass saccharification by SG2 and FS22A crude enzyme-commercial enzyme cocktail
2. LC-MS quantification of oligosaccharides
3. Production of SG2 crude enzyme in bench-scale bioreactor
4. Drafting non-provisional patent

Materials Methods:

Validation of switchgrass saccharification by SG2 and FS22A crude enzyme-commercial enzyme cocktail

Switchgrass pretreatment
Acid-pretreatment was carried out using 2% sulfuric acid. About 100g of switchgrass was soaked in 2% sulfuric acid and autoclaved at 121°C for 1h. After autoclaving the contents were allowed to cool to room temperature, liquid drained using a cheese cloth and the acid-pretreated switchgrass washed several times with water to completely remove the acid residues. After 4-5 washes the pH of the liquid was tested and was washed further if the pH was less than 5. The washed switchgrass was dried at room temperature for 72h and stored in airtight ziplock bags until further use.

Crude enzyme production
Crude enzymes of SG2 and FS22A were produced using optimized screening medium. One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution). Ten flasks per treatment containing 50ml medium were sterilized at 121°C for 1h. About 2% of 72h SG-2 or FS22A inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5th day the broth was transferred into sterile 50 centrifuge tubes, centrifuged at 5000rpm for 10 min, supernatant from respective fungi pooled and the crude enzyme was used for saccharification. The crude enzyme samples were assayed for cellulase, xylanase, beta-glucosidase and beta-xylosidase.

Enzyme assays
End of fermentation samples were analyzed for cellulase, xylanase, β-glucosidase and β-xylosidase. Method described in Saha et al (2005) with modification by Okeke and Lu (2011) was followed. Briefly, filter paper discs were used for cellulase assay. About 10 discs were

placed in glass test tubes and 0.5ml of 100mM sodium acetate buffer at pH 5.0 was added. About 0.5ml of end of fermentation sample (crude enzyme extract) was added and tubes were incubated at 50ºC for 30min. Enzyme reaction was stopped with DNS reagent and 40% sodium potassium tartarate, sample mixture placed in a boiling water bath for 10min and cooled to room temperature. Using a spectrophotometer, optical density was read at 575nm, and mg/ml of reducing sugar released was calculated using standard glucose curve. The same procedure was followed for xylanase assay except that the substrate was oats xylan.

For β-glucosidase and β-xylosidase, substrates *p*-nitrophenyl β-D-glucoside and *p*-nitrophenyl β-D-xyloside were respectively used. In brief, approximately 800µl of 100mM acetate buffer was added to 100ml of 40mM of substrate. About 100µl of crude enzyme extract was added, incubated at 50ºC for 30 min and immediately transferred onto ice cold water bath. The enzyme reaction was stopped using 1ml of chilled Na_2CO_3. Sample ODs were read at 405nm and enzyme activity expressed as micro moles of p-nitrophenol released per milliliter of enzyme extract was calculated by using standard curve.

Saccharification

Saccharification was carried out in 100ml flasks with airtight caps. About 10ml of reaction mixture was taken in each flask to which 2% of switchgrass (acid-pretreated) was added. All experiments were carried out in duplicates. Novozyme enzymes cellulase (NS22086), xylanase (NS22083), β-glucosidase (NS22118) and Hemicellulase (NS22002) were used as commercial enzyme source. Enzyme dosing was adjusted as per the recommendation by Novozymes (Units/percent total solids: cellulase 5, xylanase 0.25, β-glucosidase 0.6 and hemicellulase 2 respectively).

Enzyme treatments included (i) **C** crude aqueous enzyme of FS22A or SG-2, (ii) **C25N** FS22A or SG-2 crude extract and 25% Novozyme recommended dosage (iii) **C50N** FS22A or SG-2 crude extract and 50% Novozyme recommended dosage (iv) **C75N** FS22A or SG-2 crude extract and 75% Novozyme recommended dosage, (v) **CN** FS22A or SG-2 crude extract and Novozyme recommended dosage, (vi) **N** Novozyme enzymes recommended dosage (5% cellulase, 0.25% xylanase, 0.6% β-glucosidase and 2% hemicellulase), (vii) **25N** 25% recommended dosage of Novozyme enzymes and (viii) **50N** 50% recommended dosage of Novozyme enzymes. Saccharification was carried out at 50°C at 80rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All flasks were closed tightly to avoid escape of moisture. About 0.5ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

LC-MS quantification of oligosaccharides

Acid-pretreated switchgrass saccharified by SG2 crude enzyme alone was used to detect oligosaccharides. About 0.5ml of sample was drawn after 72h of saccharification and diluted 200 times with sterile MiliQ water and filtered with 0.2µm Phenomenex filters.

LC-MS conditions

Shimadzu LCMS 2020 equipped with dual probe ionization system (DUIS)-quadrupole mass spectrometer with two high pressure LC 20AD pump, SIL-20A autosampler and CBM-20A detector was employed for the oligosaccharide characterization. Method described by Liu et al (2005) was employed with minor modifications.

Mobile phase (50:50 of acetonitrile: 0.1% formic acid) at flow rate of 100µL/min was used. Nitrogen gas was used as nebulization and drying gas at flow rates of 1.5 and 15L/min respectively. The DL heating block temperature was maintained at 250°C and electron spray ionization (ESI) in positive mode was used. Sodium adduct was used to determine the molecular weights. About 2µL of sample was injected. Standard oligosaccharides (from Sigma Aldrich, USA) were prepared in sterile HPLC-grade water: cellobiose 1.15ppm; cellotetraose 5ppm; and cellopentaose 2.6ppm. Samples and standards were analyzed by selective ion monitoring (SIM) system. Concentrations were determined based on peak intensity.

Production of SG2 crude enzyme in bench-scale bioreactor

New Brunswick (NBS BioFlo115) 7L fermenter was used for the generation of SG2 crude enzyme. The fermenter was cleaned with water and mild detergent. In the first attempt, 5L (Fig. 9) screening medium (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution. pH varied from 5.2 to 5.35 and did not warrant any further modification) was batched and sterilized 121°C for 30min with slow exhaust. Upon cooling, 1% of *Trichoderma* SG2 inoculum was added. Dissolved oxygen (DO) was set at min. 60% to achieve high aeration and control mode was maintained in 'cascade' by air flow and stirring speed. Stirring speed was set at min. 500rpm and max. 1000rpm. Incubation at 30°C for 4d was maintained; and pH, aeration, stirring speed and air flow rate were recorded.

In the second attempt, the above method was followed except that 2L medium was taken and the fermenter (with medium) was autoclaved at 121°C for 30min on day 1, allowed to cool and autoclaved again on day 2 at 121°C for 30min and cooled. Two-step sterilization akin to Tyndallization was employed to ensure complete removal of bacterial spores.

Statistical analyses

When required data were statistically analyzed using Statistical Analysis Software (SAS version 9.2). *PROC GLM* was used to compare treatments and pair-wise differences were detected by TUKEY with significance set at $P=0.05$ (Treatments that share a letter are not significantly different from each other).

Results and Discussion

Validation of switchgrass saccharification by SG2 and FS22A crude enzyme-commercial enzyme cocktail:

The enzyme activities of SG2 and FS22A are provided in Table 1.

Overall data analysis indicated that enzyme treatment was significant ($P<0.0001$):
Glucose and xylose yield: $FS22A^{a}>SG2^{b}>Novozyme^{c}$

Comparison of FS22A and Novozyme enzyme:
Glucose yield ($P<0.0001$): $C75N^{a}>CN^{a}>C50N^{a}>C25N^{a}>C^{b}>N^{b}>50N^{c}>25N^{c}$
Xylose yield ($P<0.0001$): $CN^{a}>C75N^{ab}>C50N^{ab}>C25N^{b}>C^{c}>N^{c}>50N^{d}>25N^{d}$

Comparison of SG2 and Novozyme enzyme:
Glucose yield ($P<0.0001$): $CN^{a}>C75N^{ab}>C25N^{bc}>C50N^{cd}>C^{de}>N^{e}>50N^{f}>25N^{g}$

Xylose yield (*P<0.0001*): $C75N^{a}$>CN^{a}>$C25N^{ab}$>$C50N^{b}$>C^{b}>N^{bc}>$50N^{c}$>$25N^{c}$

Comparison of respective enzyme combinations in FS22A and SG2:
Overall glucose and xylose yields (P<0.05): $FS22A^{a}$>$SG2^{b}$
Glucose yield: C: $SG2^{a}$>$FS22A^{a}$
C25N: $FS22A^{a}$>$SG2^{a}$
C50N, C75N and CN: $FS22A^{a}$>$SG2^{b}$
Xylose yield: C: $SG2^{a}$>$FS22A^{a}$
C50N: $FS22A^{a}$>$SG2^{b}$
C25N, C75N and CN: $FS22A^{a}$>$SG2^{a}$

This shows that even though sugar yields from crude enzyme alone **C** of SG2 and FS22A are not statistically different (ie., they yield similar amount of sugars), their combinations with different concentrations of commercial enzyme **N** result in significantly different sugar yields.

Within each time point the respective treatments for FS22A and SG2 were compared (P<0.0001; Figs. 3 and 4). For glucose yield, all treatments at 72h were significantly different and at 48h all treatments except C25N were significantly different. At 24h, all treatments were similar except CN. For xylose yield, at 72h all treatments were similar and at 48h and 24h all treatments were similar except C50N and C25N respectively.

The switchgrass sample used in this experiment had about 42% cellulose and acid-pretreated sample had about 60% cellulose respectively (Table 2). Accordingly, the percent conversion efficiency of glucose from cellulose is provided in Table 3. Crude enzymes of both FS22A and SG2 were 35% and 30% respectively and that of Novozyme enzyme was 28%. Overall, the crude enzyme-commercial enzyme combinations of SG2 resulted in 30 to 39% conversion efficiency whereas it was 35% to 49% for FS22A respectively.

Irrespective of time point, C50N of FS22A yielded significantly higher glucose than commercial enzyme N alone, and is not significantly different from C75N or CN. In the case of SG2, C50N yielded significantly higher sugar than commercial enzyme N, and significantly lesser glucose than C75N or CN.

LC-MS quantification of oligosaccharides
The 72h-SG2-saccharified 2% acid-pretreated switchgrass sample yielded 13.45ppm cellobiose, 166.67ppm cellotetraose and 240ppm cellopentaose respectively (Figs. 5-7). Fig. 8 is the overall spectral analysis 72h-SG2-saccharified 2% acid-pretreated switchgrass sample and majority of oligosaccharides in the sample were found between 300-900 mass units.

The switchgrass sample used in this study contained 60% cellulose which translated into 12g/L cellulose and with 40% glucose conversion efficiency the relative abundance after 72h saccharification was cellobiose 0.1%, cellotetraose 1.4% and 2% cellopentaose respectively.

Production of SG2 crude enzyme in bench-scale bioreactor
In the first attempt after 48h, based on pH and also due to sampling, bacterial contamination was detected. The contamination persisted in the second attempt and it was found that the 0.2µm filter was faulty. Since the filter was an add-on, a replacement has been requested.

Drafting non-provisional patent

A non-provisional U.S. Patent titled ***An Efficient Process For Producing Ethanol From A Biomass Feedstock*** was drafted and the final application with the inputs of the patent attorneys has been compiled. In accordance with Patent law, the microbial cultures isolated and used in this study (*Trichoderma* SG2, SG4, FS5A and *Fusarium* FS22A) will have to be deposited in a culture collection center. The process has been initiated and the cryo-vials of these cultures will be deposited with American Type Culture Collection (ATCC, Manassas, VA).

Table 1 Enzyme activities (U/ml/30min) in crude enzyme (C) of SG2 and FS22A. Means and standard errors are reported.

Enzyme	*FS22A*	*SG2*
Cellulase	6.29±0.004	8.16±0.005
Xylanase	40.64±0.001	37.96±0.012
Beta-Glucosidase	0.2±0.001	13.1±0.005
Beta-Xylosidase	1±0.001	6.3±0.066

Crude enzyme produced after 5d submerged fermentation in shake-flasks

Table 2 Compositional analyses of feedstock samples used in this project

W/W%	Crude protein	Crude fiber	ADF	NDF	Ash	Cellulose	Lignin	Hemicellulose	Reducing Sugars
Union Springs (old) switchgrass	6.31	33.95	40.52	78.30	4.76	33.89	5.75	37.78	3.07
Acid-treated Union Springs (old) switchgrass	5.30	48.90	64.96	76.06	2.44	53.12	10.15	11.10	0.47
Auburn (new) switchgrass	2.55	43.31	51.69	83.53	1.48	41.59	9.97	31.84	1.42
Acid-treated Auburn (new) switchgrass	1.89	60.00	82.30	84.63	0.52	59.34	22.87	2.33	0.40

Table 3 Percent conversion efficiency of cellulose to glucose in 2% acid-pretreated switchgrass in shake-flasks

Enzyme	**Fungal strain**	
Fungal/Commercial cocktail	*FS22A*	*SG2*
C	35	30
C25N	39	33
C50N	41	35
C75N	49	36
CN	45	39
Commercial		
N	28	
25N	16	
50N	22	

C Crude enzyme (*Fusarium* FS22A or *Trichoderma* SG2)
C25N Crude enzyme of FS22A or SG2 supplemented with 25% of recommended commercial (Novozyme) enzyme dose
C50N Crude enzyme of FS22A or SG2 supplemented with 50% of recommended commercial (Novozyme) enzyme dose
C75N Crude enzyme of FS22A or SG2 supplemented with 75% of recommended commercial (Novozyme) enzyme dose
CN Crude enzyme of FS22A or SG2 supplemented with recommended commercial (Novozyme) enzyme dose
N recommended commercial (Novozyme) enzyme dose
25N 25% of recommended commercial (Novozyme) enzyme dose only
50N 50% of recommended commercial (Novozyme) enzyme dose only

Table 4 Comparison of percent glucose conversion efficiency from 2% acid-pretreated switchgrass (shake-flasks only) by FS22A and SG2

Enzyme cocktail	Fungus				
	FS22A		*SG2*		
	Mar 2013^	Jul 2012*	Mar 2013^	Jul 2012*	Jan 2012*
C	35	41	30	28	36
C25N	39	54	33	51	48
C50N	41	67	35	50	53
C75N	49		36		
CN	45		39		
N	28	38	28	38	41
25N	16	21	16	21	
50N	22	31	22	31	

* Union springs (old) switchgrass (see compositional analyses in Table 2)
^ Auburn (new) switchgrass (see Table 2)

Figures

Figure 1

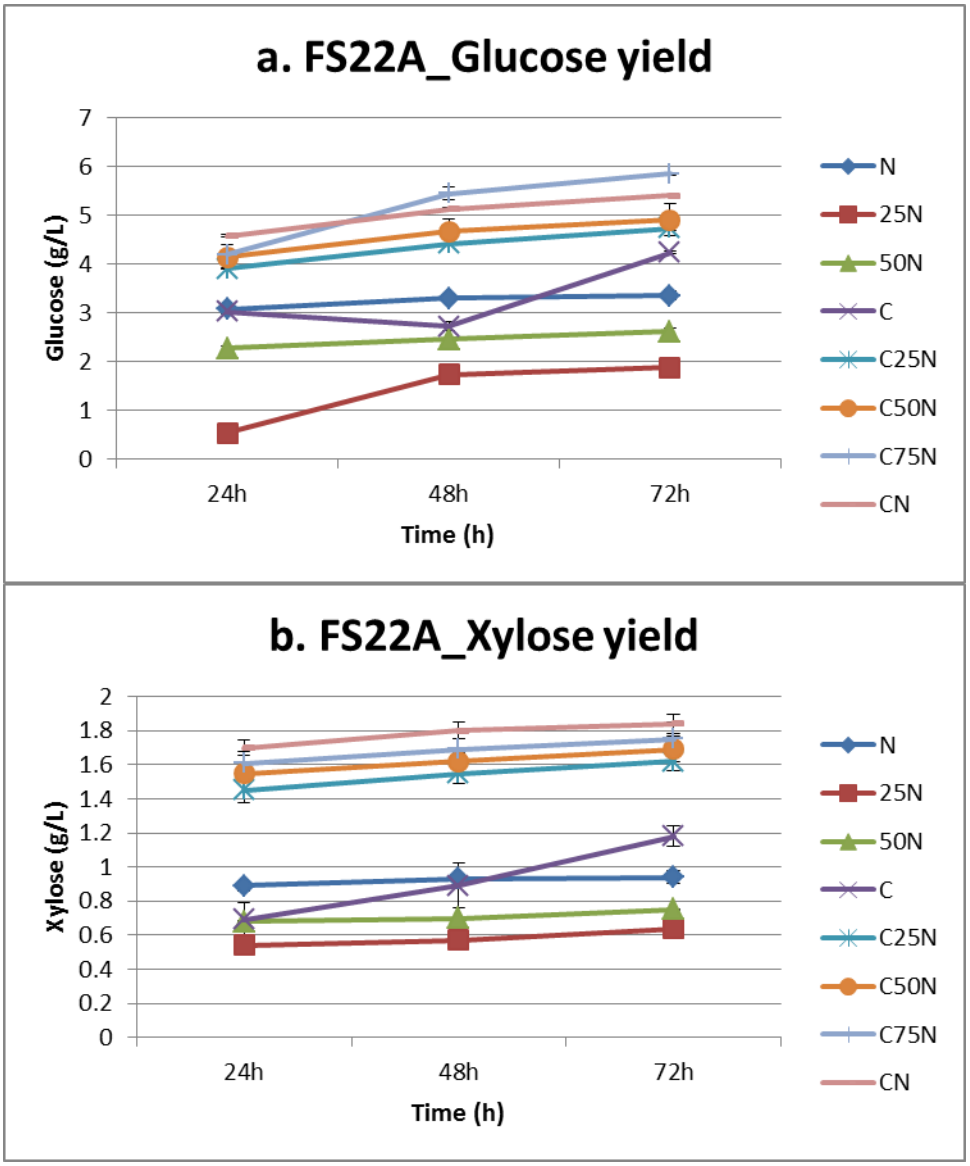


Figure 45 Sugar yield by *Fusarium* FS22A crude enzyme. a. Glucose b. Xylose. Means and standard errors are indicated.

Figure 2

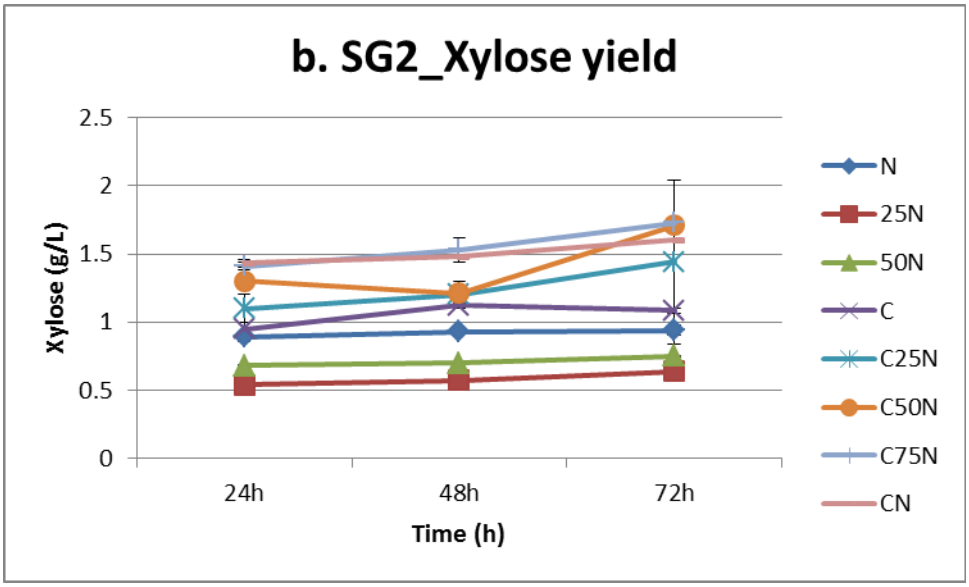


Figure 46 Sugar yield by *Trichoderma* SG2 crude enzyme. a. Glucose b. Xylose. Means and standard errors are indicated.

Figure 3

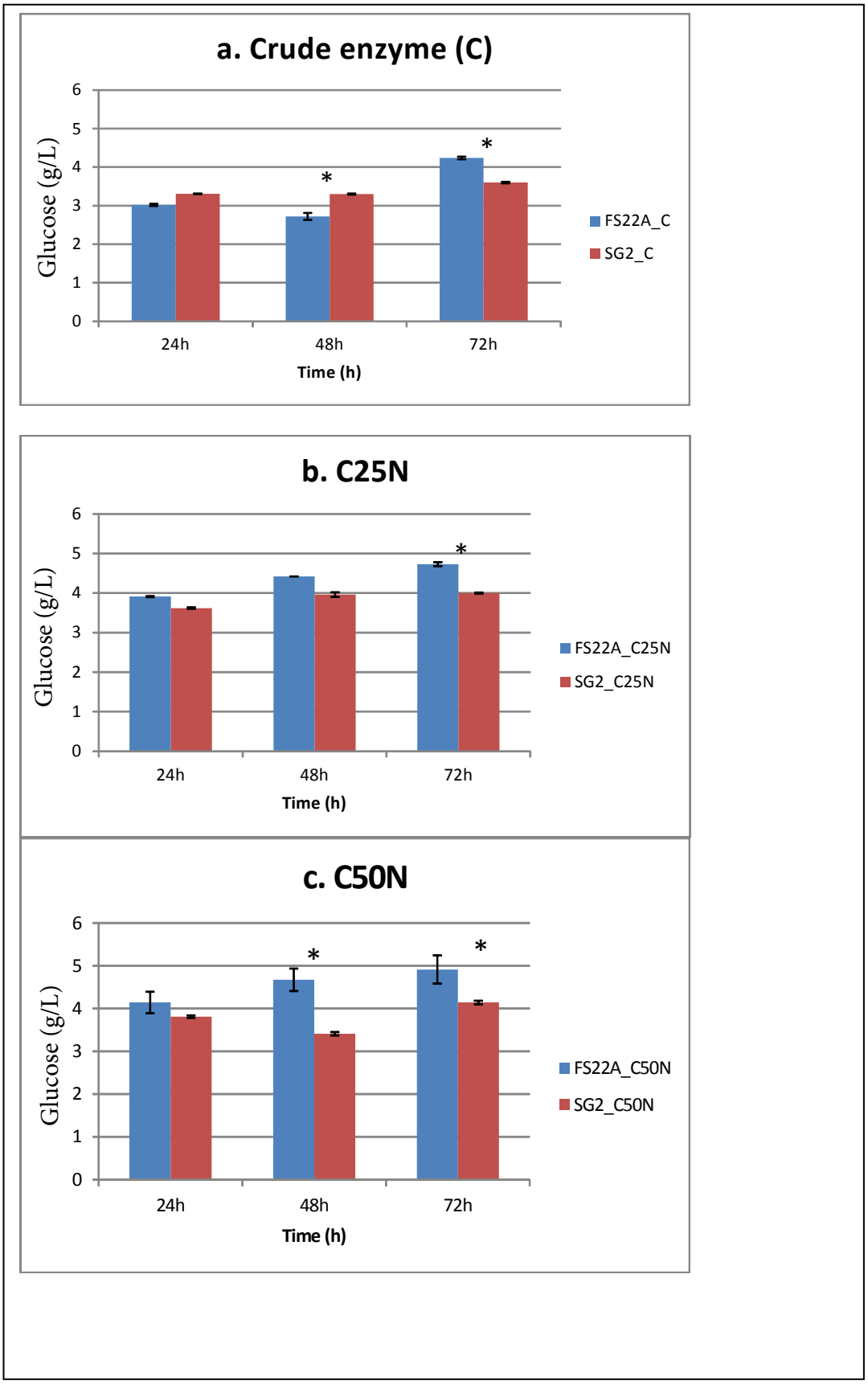
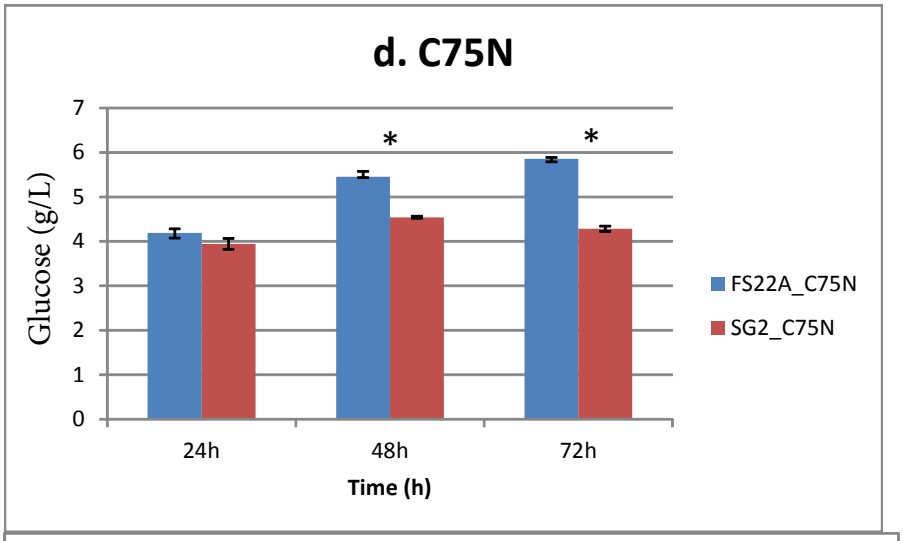

a. Crude enzyme (C)
Glucose (g/L)
0
1
2
3
4
5
6
24h
48h
72h
Time (h)
*
*
FS22A_C
SG2_C
b. C25N
Glucose (g/L)
0
1
2
3
4
5
6
24h
48h
72h
Time (h)
*
FS22A_C25N
SG2_C25N
c. C50N
Glucose (g/L)
0
1
2
3
4
5
6
24h
48h
72h
Time (h)
*
*
FS22A_C50N
SG2_C50N

Figure 3 contd.



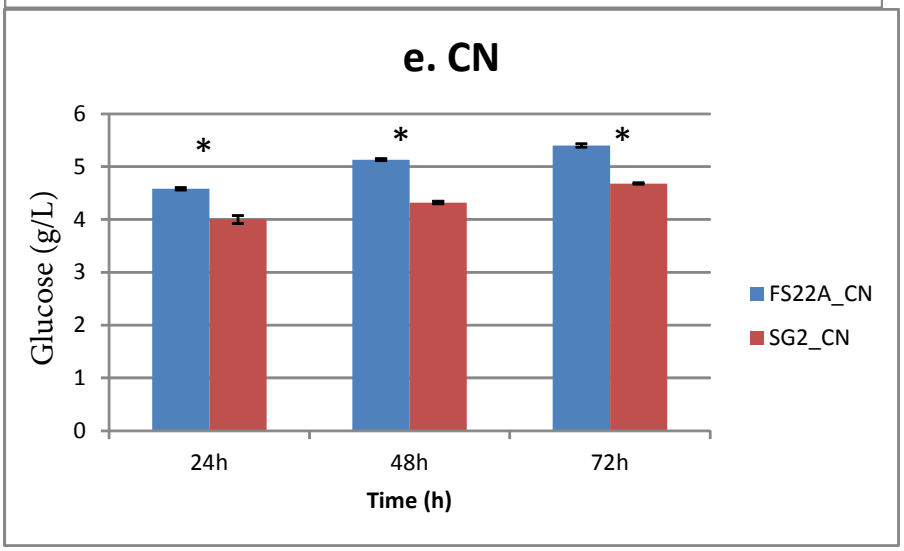


Figure 47 Comparison of glucose yield from FS22A and SG2. Means and standard errors are indicated. Significant ($P<0.05$) pair-wise differences represented by *.

Figure 4

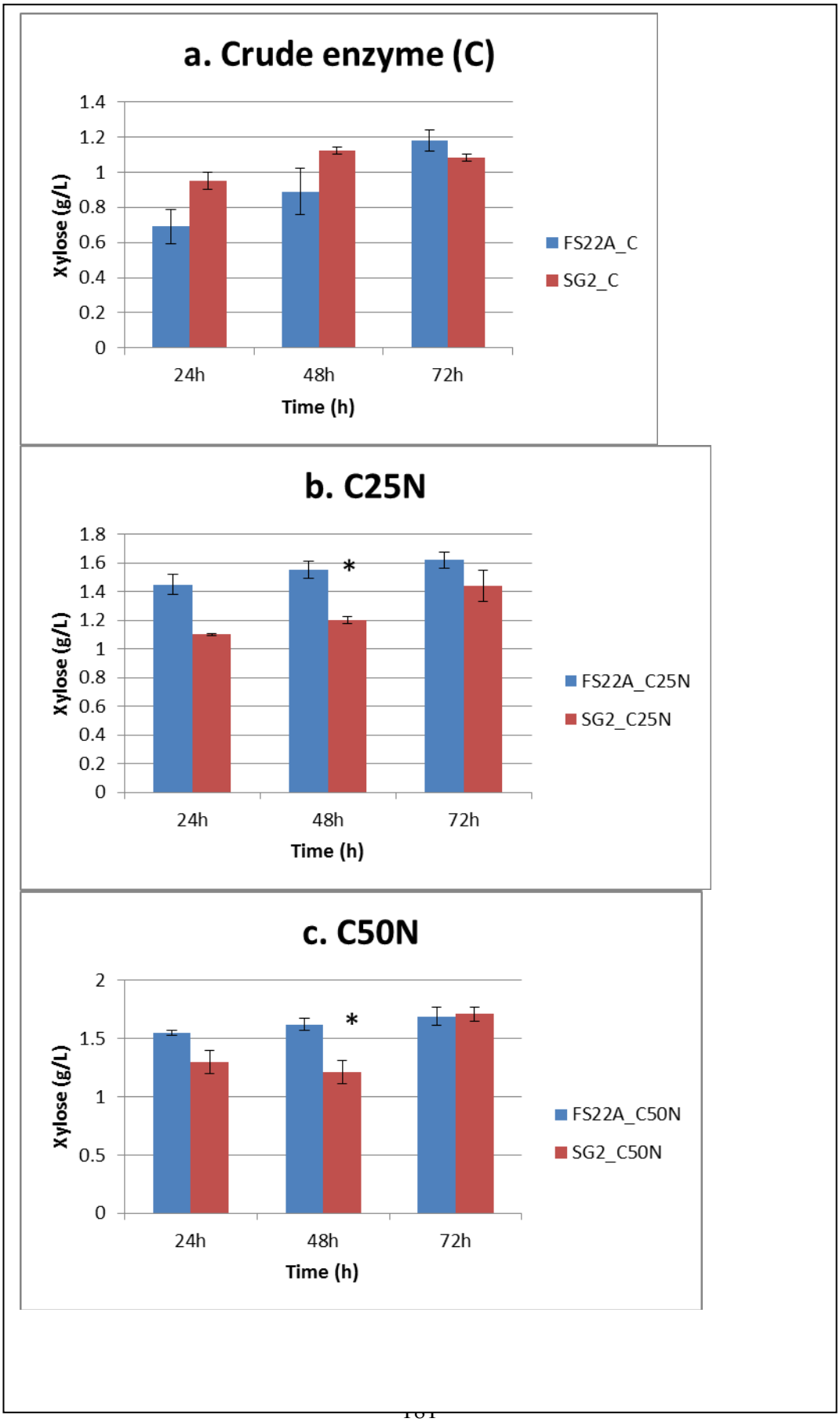
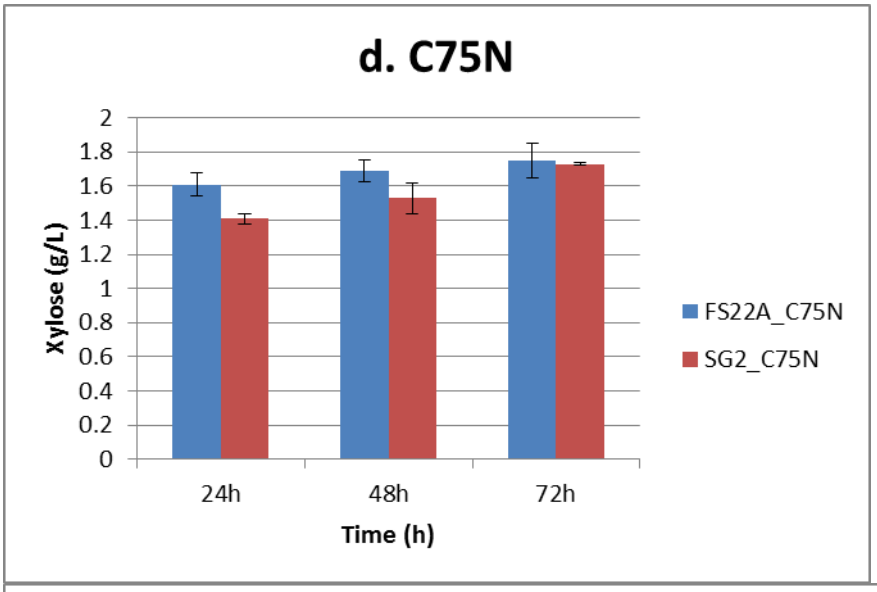

a. Crude enzyme (C)
Xylose (g/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24h
48h
72h
Time (h)
FS22A_C
SG2_C
b. C25N
Xylose (g/L)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
24h
48h
72h
Time (h)
FS22A_C25N
SG2_C25N
c. C50N
Xylose (g/L)
2
1.5
1
0.5
0
*
24h
48h
72h
Time (h)
FS22A_C50N
SG2_C50N

Figure 4 contd.



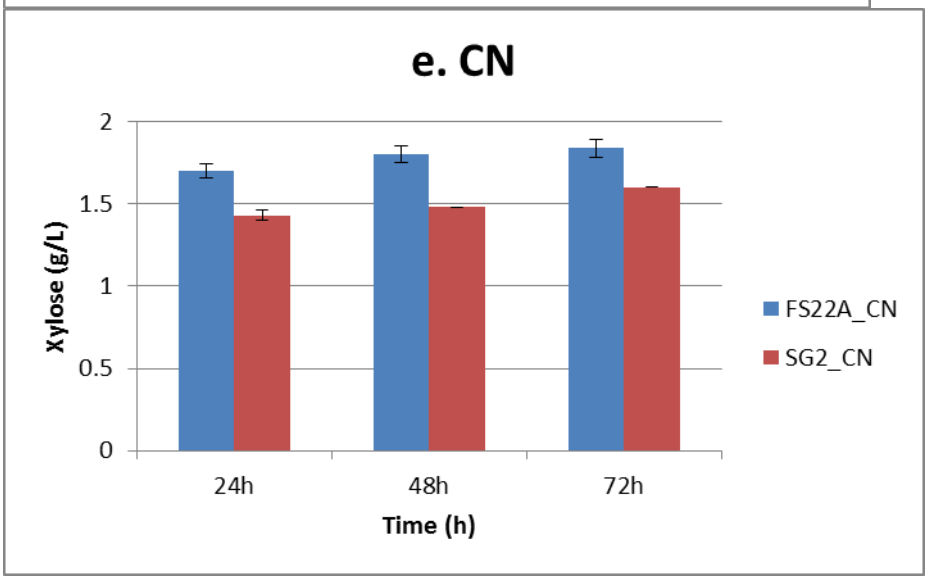
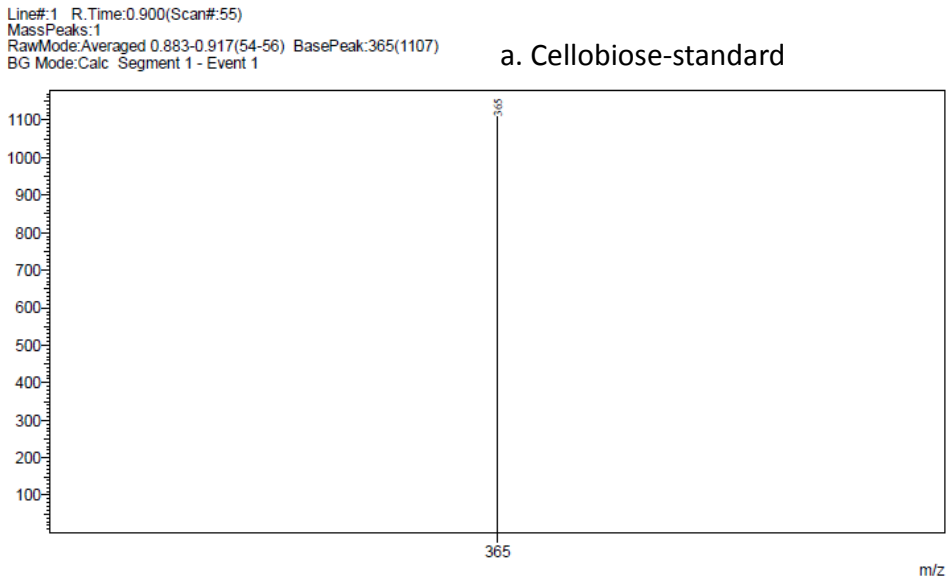


Figure 48 Xylose yield comparison of FS22A and SG2. Means and standard errors are provided. Significant ($P<0.05$) pair-wise differences indicated by *.

Figure 5



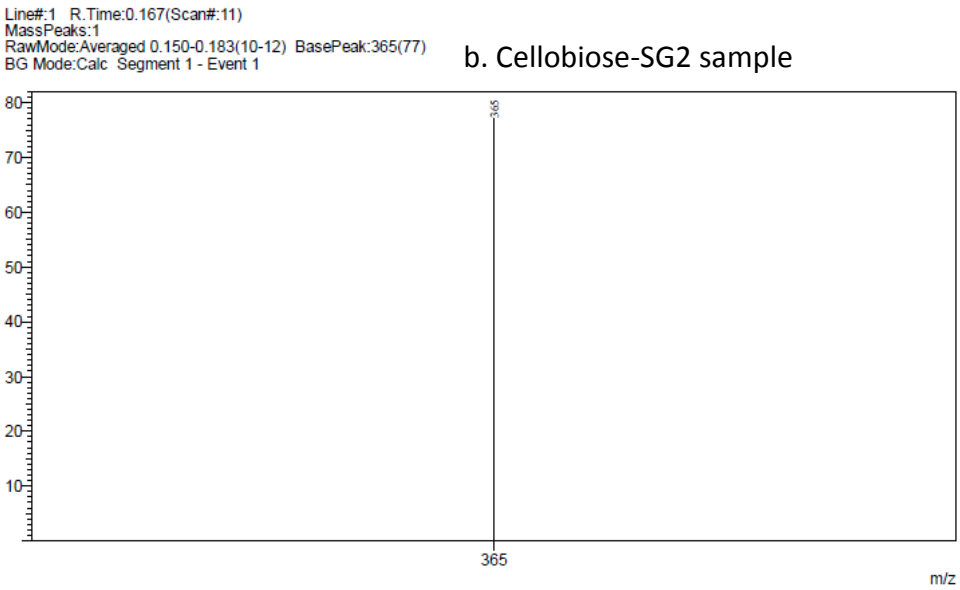


Figure 49 LC-MS of cellobiose. a. Standard b. SG2 crude enzyme-saccharified 72h sample of 2% acid-pretreated switchgrass.

Figure 6

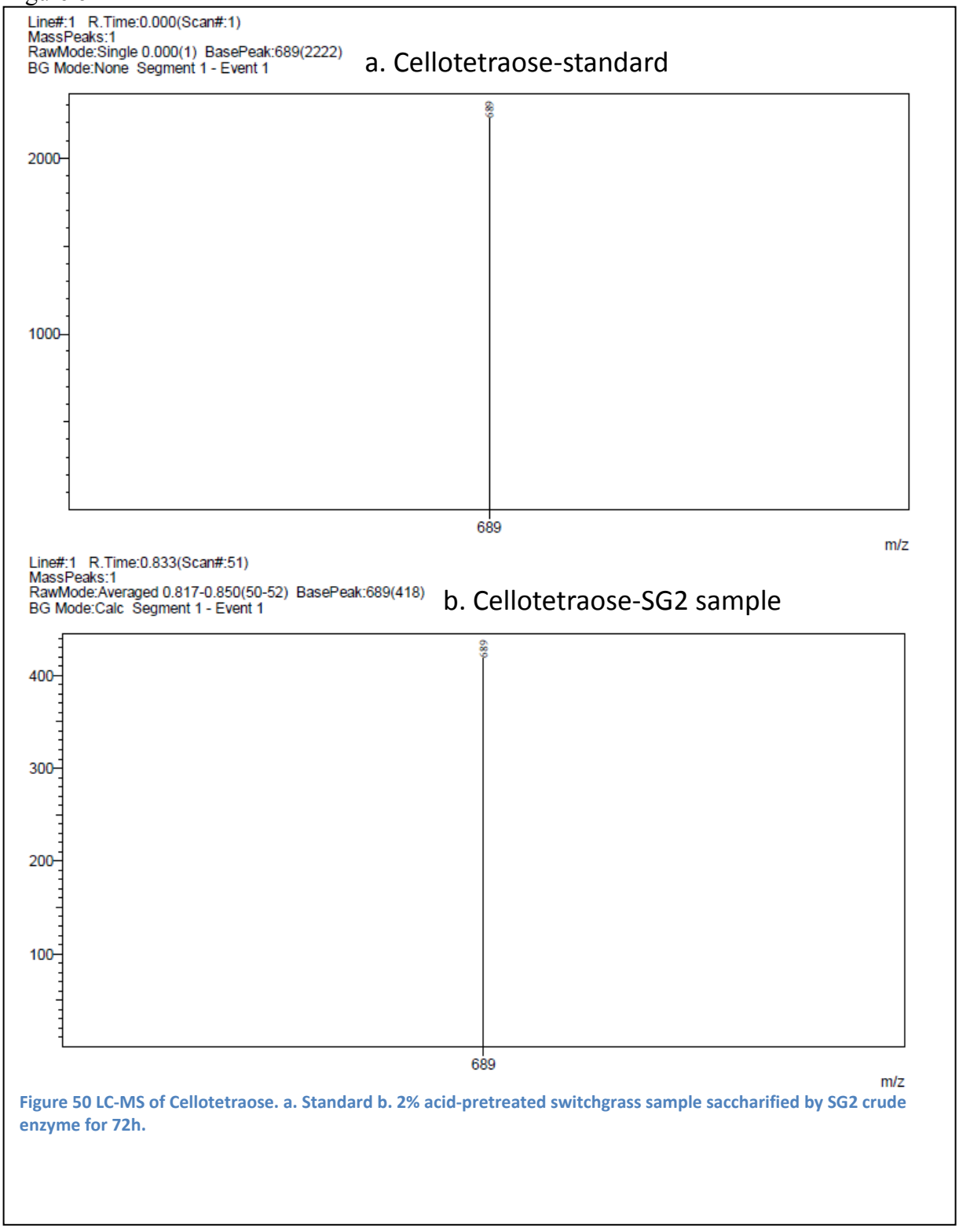


Figure 50 LC-MS of Cellotetraose. a. Standard b. 2% acid-pretreated switchgrass sample saccharified by SG2 crude enzyme for 72h.

Figure 7

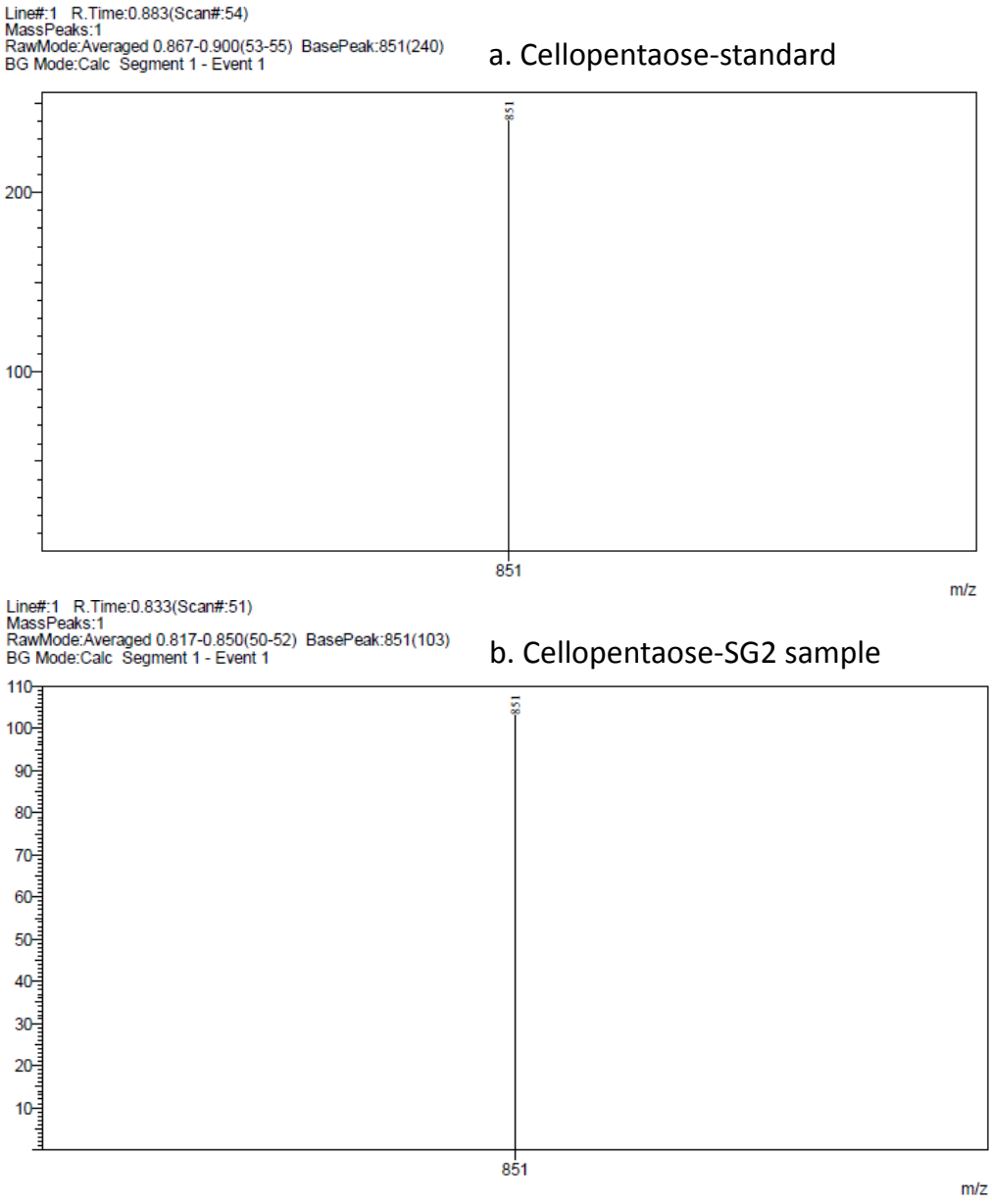


Figure 51 LC-MS of cellopentaose. a. Standard b. 2% id-pretreated switchgrass sample saccharified by SG2 crude enzyme for 72h.

Figure 8

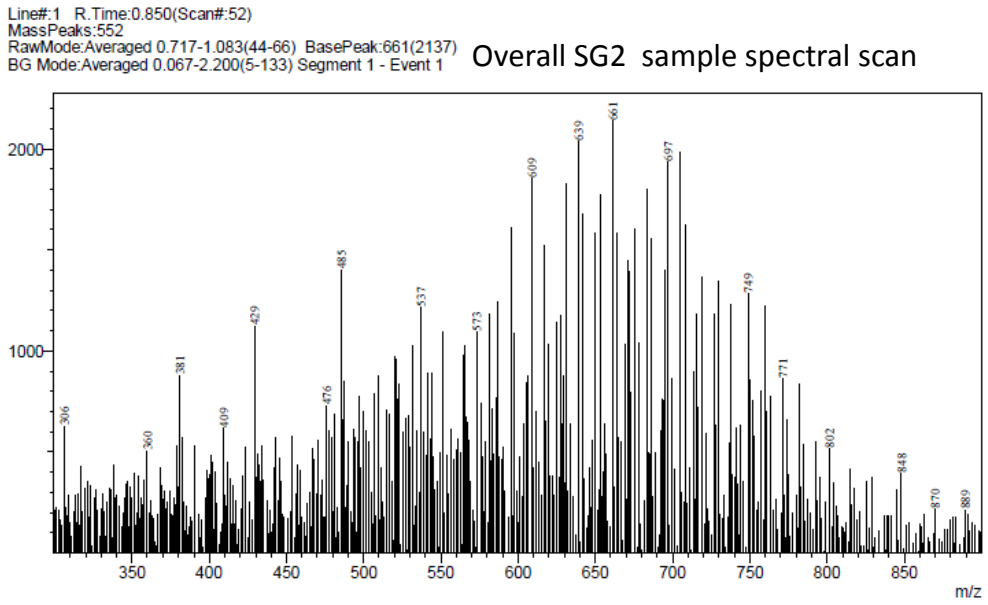


Figure 52 LC-MS scan of 2% acid-pretreated switchgrass saccharified by SG2 crude enzyme for 72h (300-900 mass units).

Figure 9

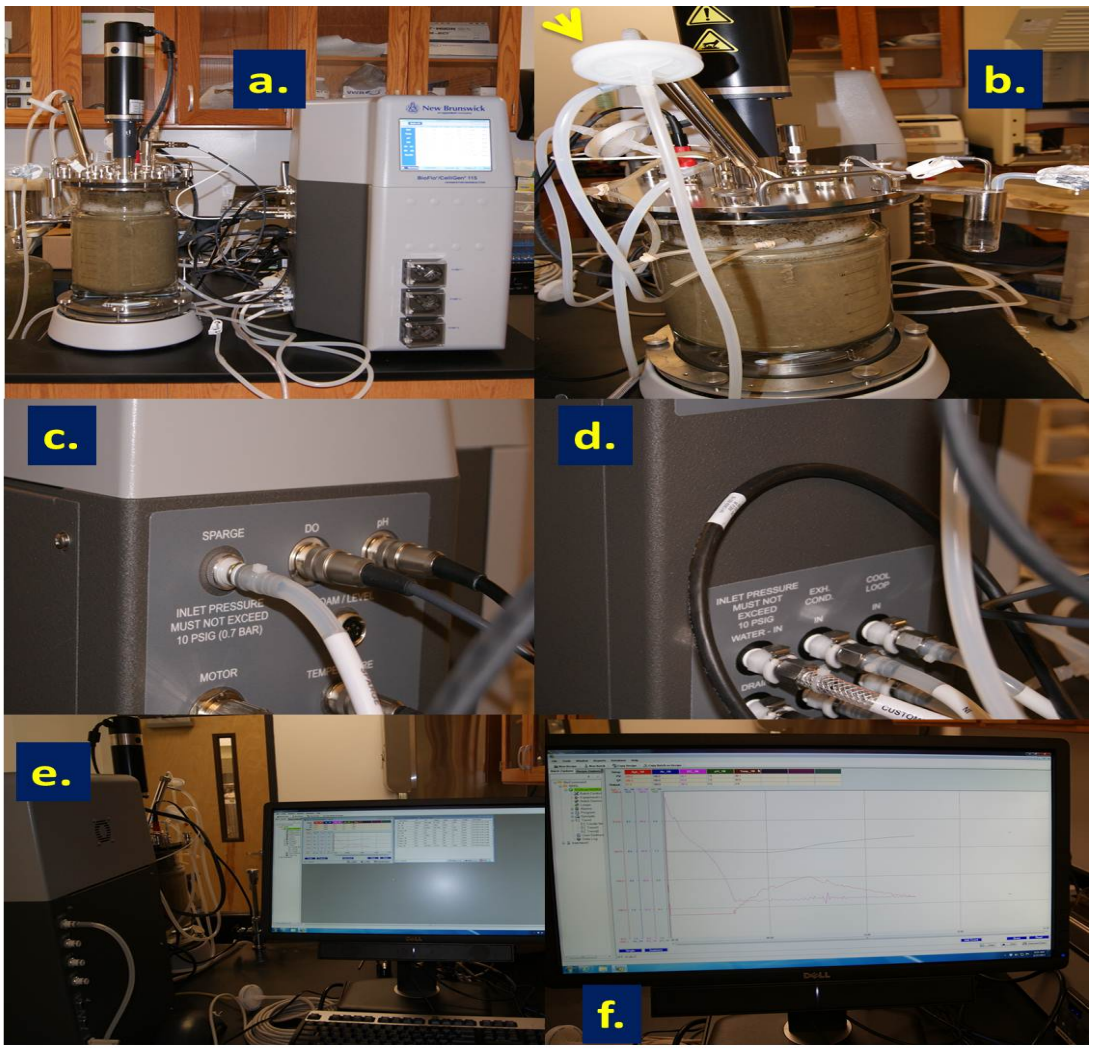


Figure 53 Bench-top New Brunswick NBS BioFlo115 7L fermenter. a. 5L SG2 fermentation b. air filter fitted to fermenter c & d. ports e & f. data acquisition. Arrow points to the air filter.

Figure 10

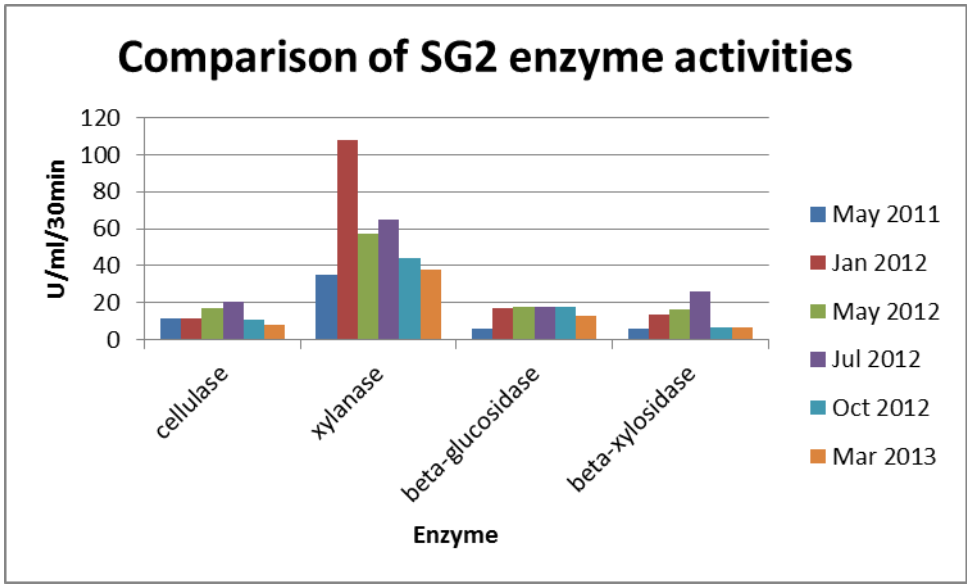


Figure 54 Comparison of few SG2 crude enzyme activities recorded over the years.

Conclusions and Milestones

Validation of switchgrass saccharification by SG2 and FS22A crude enzyme-commercial enzyme cocktail

- The crude enzyme-commercial enzyme cocktail has been evaluated three times previously and the treatments included were **C**, **C25N** and **C50N**. In this experiment, two more treatments, **CN** and **C75N** were included to broaden the range of enzyme combinations.
- In FS22A, using 2% acid-pretreated switchgrass, **C50N** resulted in 47% more glucose, **C75N** resulted in 75% more glucose, and **CN** resulted in 61% more glucose than commercial enzyme **N** alone.
- Similarly in SG2, **C50N** resulted in 24% more glucose, **C75N** resulted in 28% more glucose and **CN** resulted in 40% more glucose than commercial enzyme alone.
- Overall in comparison to commercial enzyme **N** alone, **C50N** of FS22A is ideally suited for switchgrass saccharification. Whereas in SG2, **C25N** or **C50N** can be used for switchgrass saccharification.
- Based on the data from 2011, 2012 and 2013 (Table 3), we find that the C25N and/or C50N of either FS22A or SG2 result in higher glucose conversion efficiency from cellulose in switchgrass than N alone. So, despite the variation in enzyme activities (Fig. 10 and compositional variability in switchgrass samples (Table 2), the trend remains the same. By expanding the combinations of crude and commercial enzyme (C75N and CN), only SG2 resulted in significantly greater sugar yield than C50N.
- It is concluded that using crude enzyme **C** of either SG2 or FS22A instead of water in the saccharification of 2% acid-pretreated switchgrass supplemented with 25% to 100% of recommended commercial (Novozyme) results in higher sugar yield than using commercial enzyme alone.

LC-MS quantification of oligosaccharides

- Previously (second quarterly report 2012), the qualitative assessment of oligosaccharides by LC-MS in 24h and 72h 2% acid-pretreated switchgrass-saccharified by SG2 (C, C25N and C50N) and commercial enzyme N found the presence of cellobiose, cellotriose, celotetraose and cellopentaose.
- In this report, oligosaccharides resulting after 72h saccharification of 2% acid-pretreated switchgrassby by SG2 crude enzyme C were quantified and based on the detection of oligosaccharides (13.45ppm cellobiose, 166.67ppm cellotetraose and 240ppm cellopentaose respectively), their relative abundance was found to be 0.1% cellobiose, 1.4% cellotetraose and 2% cellopentaose.
- SG2 crude enzyme saccharification of acid-pretreated switchgrass results in low concentration of cellobiose and greater abundance of larger oligosaccharide.
- Oligosaccharide profiling can assist in improving saccharification efficiency.

References:

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PART 10: Research Activities of FY-12 (April 1, 2013 to June 31, 2013
Written by: Ananda Nanjundaswamy and Benedict Okeke

Introduction:
In the previous quarter, validation of switchgrass saccharification by SG2 and FS22A crude enzyme-commercial enzyme cocktail was carried out. It was consistently found that in comparison to commercial enzyme **N** alone, **C50N** (crude culture mixed with **50**% recommended commercial enzyme dosing) of FS22A is ideally suited for switchgrass saccharification whereas **C25N** (crude culture mixed with 2**5**% recommended commercial enzyme dosing) or **C50N** of SG2 can be used for switchgrass saccharification. Based on the data from 2011, 2012 and 2013, we found that the C25N and/or C50N of either FS22A or SG2 result in higher glucose conversion efficiency from cellulose in switchgrass than commercial enzyme N alone. It was concluded that using crude enzyme **C** of either SG2 or FS22A instead of water in the saccharification of 2% acid-pretreated switchgrass supplemented with 25% to 100% of recommended commercial results in highest sugar yield than using commercial enzyme alone. LC-MS quantification of oligosaccharides in SG2 crude enzyme saccharified-acid-pretreated switchgrass resulted in low concentration of cellobiose and greater abundance of larger oligosaccharide. In this quarter the following objectives were addressed:

Specific objectives
1. Evaluation of different pretreatment strategies.
2. Drafting of non-provisional patent and filling of provisional patent (US 61/787,039) pending completion of the non-provisional patent.

Materials Methods:

Evaluation of different pre-treatment strategies
At least eight different pretreatment strategies were evaluated for switchgrass: 0.5% acid only; 0.5% acid followed by 0.5% acid; 2% acid only; 2% alkali only; 2% acid followed by 2% alkali; 2% alkali followed by 2% acid; 85% acid followed by acetone; and 85% acid followed by ethanol. In all our previous experiments, it was found that 2% acid (sulfuric acid alone) was found to be the ideal pretreatment method and in this experiment was used as a control.
Switchgrass pretreatment:

For pretreatments involving 0.5% acid and 2% acid or 2% alkali, sulfuric acid and sodium hydroxide were used respectively. About 100g of switchgrass was soaked in respective acid or alkali solution and autoclaved at 121°C for 30min with slow exhaust. After autoclaving the contents were allowed to cool to room temperature, liquid drained using a cheese cloth and the pretreated switchgrass washed several times with water to completely remove any residues. After 4-5 washes the pH of the liquid was tested and was washed further if the pH was less than 5. The washed switchgrass was dried at room temperature for 72h and stored in airtight ziplock bags until further use.

For acid followed by alkali pretreatment (Acid→Alkali), 10% switchgrass was soaked in 2% sulfuric acid and autoclaved at 121°C for 30 min. After autoclaving samples were washed several times with running water until the pH reached 5-6. Samples were then soaked in 2% sodium hydroxide and autoclaved at 121°C for 30 min. After autoclave the samples were washed thoroughly until the pH reached 5-6 range and the samples were air dried at

room temperature for 72h. Similar procedure was followed for 2% alkali followed by 2% acid (Alkali→Acid) in the sequential manner.

For sequential mild acid pretreatment, samples were first treated with 0.5% sulfuric acid as outlined above and followed by second pretreatment with 0.5% acid after removing first residue.

For phosphoric acid pretreatment, modifications to the methods described in Zhang et. al. (2007) and Sathitsuksanoh et al (2012) were followed. Briefly, 13.125g of switchgrass was mixed with 100ml of 85% phosphoric acid and maintained at 50°C in a water bath at 1 atmosphere pressure for 60min. The reaction was terminated by adding 250ml of 95% ethanol. The samples were centrifuged at 4500rpm at room temperature for 10min. The pellet was re-suspended in 250ml of 95% ethanol, followed by centrifugation at 4500rpm for 10 min and the pellet was washed with deinozation water 3-4 times until the pH reached 5-6. The same method was carried out with 85% phosphoric acid followed by pure acetone instead of ethanol.

Crude enzyme production

Crude enzyme of *Trichoderma* SG2 was produced using optimized screening medium. (One liter medium: 6.2g powdered waste paper, 9.6g pulverized switch grass, 1.4g peptone, 0.6g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution). Ten flasks per treatment containing 50ml medium were sterilized at 121°C for 1h before inoculation. About 2% of 72h SG-2 inoculum was added into each flask aseptically. Flasks were incubated at 30°C for 5 days. On 5^{th} day the broth was transferred into sterile 50 centrifuge tubes, centrifuged at 5000rpm for 10 min, supernatant from respective fungi pooled and the crude enzyme was used for saccharification. Enzyme activities were determined as previously described (Okeke and Lu, 2011).

Saccharification

Saccharification was carried out in 100ml flasks with airtight caps. About 10ml of reaction mixture was taken in each flask to which 2% of switchgrass (pretreated) was added. All experiments were carried out in duplicates. **C** crude aqueous enzyme of *Trichoderma* SG-2 was used for saccharification carried out at 50°C at 80 rpm mixing using a Thermos Scientific MaxQ 4000 shaker. All flasks were closed tightly to avoid escape of moisture. About 0.5ml of sample was drawn at 24h interval up to 72h. Samples were subjected to sugar quantification using HPLC (Ananda et al 2011).

Results and Discussion

Evaluation of different pre-treatment strategies

Saccharification of switchgrass with different pretreatments is outlined in Fig.1. As far as glucose yield after 72h saccharification by SG2 crude enzyme was concerned, the highest yield of 5.4g/L was obtained in 85% phosphoric acid-acetone treatment > 2% alkali-2% acid > 85% acid-alcohol>2%acid-2% alkali >0.5% acid-0.5% acid> 2% acid alone>0.5% acid alone > 2% alkali alone.

Interestingly, both 85% phosphoric acid-alcohol method and 2% alkali-2% acid yielded around 4.6g/L sugar. The previously optimized pretreatment for switchgrass was 2% sulfuric acid alone which yielded 2g/L sugar upon saccharification by SG2 crude enzyme.

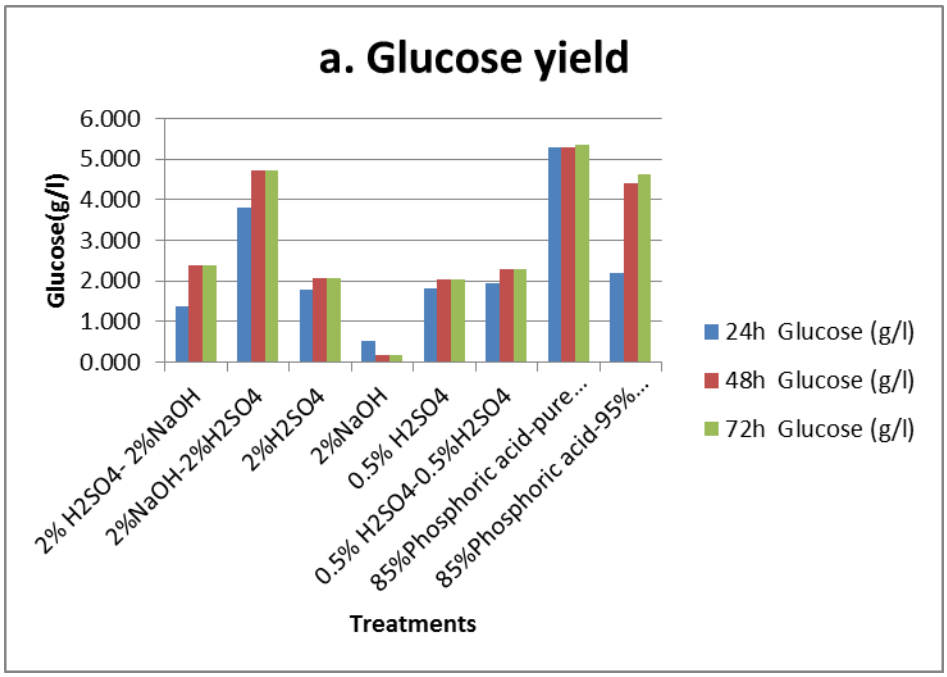
As far as xylose yield was concerned (Fig. 1b), the highest sugar yield of 2.4g/L was found in 2% alkali-2% acid treatment followed by 2% alkali alone > 0.5% acid alone > 0.5% acid-0.5% acid > 85% acid-acetone > 2% acid alone > 85% acid-alcohol > 2% acid-2% alkali.

Drafting non-provisional patent

A U.S. Provisional Patent US 61/787,039 titled ***An Efficient Process For Producing Ethanol From A Biomass Feedstock*** was drafted with the inputs of patent attorneys and filed. The application is being updated with further work for non-provisional filling. In accordance with patent law, a representative microbial culture for the process (*Trichoderma* SG2) was deposited (PTA-120389) with American Type Culture Collection (ATCC), Manassas, VA; PTA-120389.

Previously, U.S. Provisional Patent (Application No. 61/546,831) titled ***Farm-deployable bioreactor system for self-sufficient production of fuel ethanol using novel biomass-degrading microbes*** by inventors Okeke BC and Nanjundaswamy AK was filed on October 13, 2011.

Figure 1



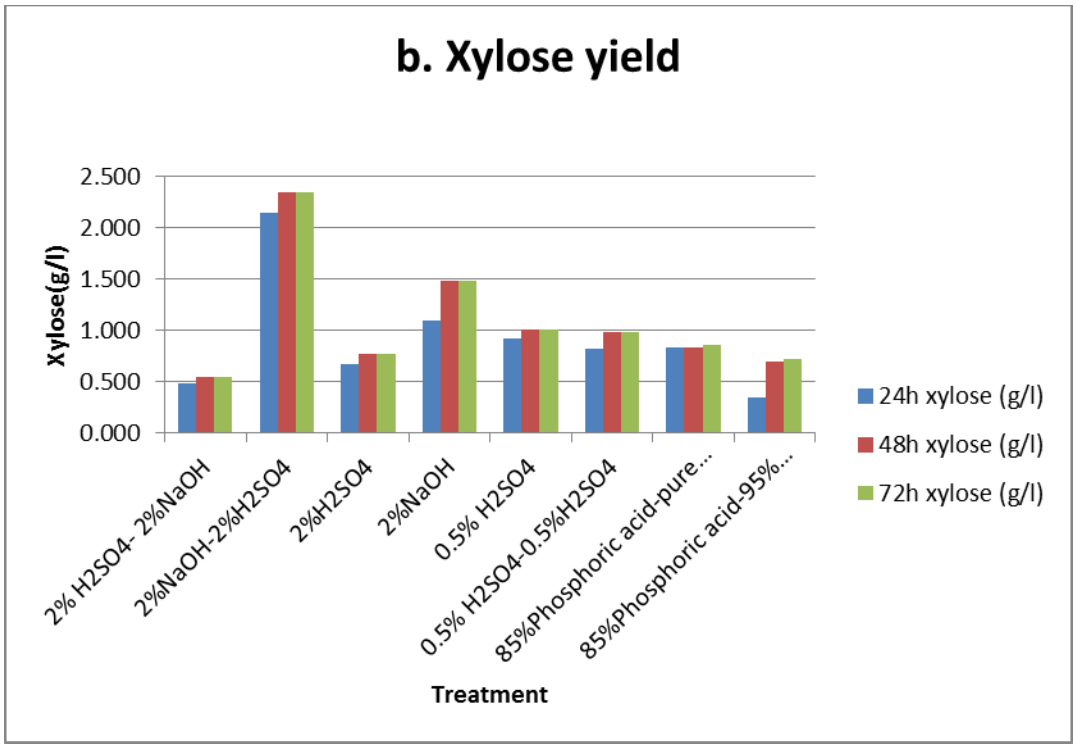


Figure 55 Sugar yield from 2% switchgrass subjected to different pretreatments. a. glucose b. xylose.

Conclusions and Milestones:

Evaluation of different pre-treatment strategies

- The most widely followed pretreatment methods involve mild acid or alkali (2% sulfuric acid or 2% sodium hydroxide). Initially, in this project we found that 2% sulfuric acid was ideal for the pretreatment of switchgrass.
- In this quarter we developed different combinations of acid and alkali pretreatments (2% sulfuric acid, 2% sodium hydroxide), very mild acid (0.5% sulfuric acid) and concentrated acid pretreatments (85% phosphoric acid followed by alcohol or acetone).
- 85% phosphoric acid followed by acetone resulted in 5.4g/L glucose and 85% phosphoric acid followed by acetone resulted in 4.6 g/L glucose. However, very low concentration of acid and alkali ie., 2% sodium hydroxide followed by 2% sulfuric acid yielded 4.7g/L glucose and the highest xylose yield of 1.5g/L.
- It is therefore concluded that very low concentration of alkali followed by very low concentration of acid results in high sugar yield and comparatively, the 85% phosphoric acid treatment gives only 15% higher sugar yield.
- The use of 1L of 85% phosphoric acid and nearly 5L of acetone for pretreatment of 10% switchgrass seems excessive compared to 1L 2% sodium hydroxide and 1L 2% sulfuric acid for pretreatment of 10% switchgrass for only 15% greater sugar yield.
- It can be concluded that 2% alkali followed by 2% acid is ideal from economical stand point and from the perspective of environmental safety.

Presentations

1. Nanjundaswamy A, Starr C and Okeke B. Processing of Biomass-Saccharifying Enzymes by Sand Filtration for 'Farm Deployable Microbial Bioreactor' Laboratory Model. 2013, American Society for Microbiology, 113th General Meeting, May 18-21, 2013 in Denver, Colorado.

2. Nanjundaswamy A and Okeke B. Potential cost reduction of cellulosic biomass saccharification by fungal crude enzyme–commercial enzyme cocktail. TechConnect World Conference and Expo 2013, May 12-16, 2013, National Harbor, Maryland.

3. Nanjundaswamy A and Okeke B. Development of Fungal Crude Enzyme: Commercial Enzyme Cocktail for Potential Cost Reduction of Cellulosic Biomass

Saccharification. 29th Annual Fuel Ethanol Workshop, June 10-13, 2013, St. Louis, MO (Talk).

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PART 11: Research Activities of FY-13(July 1, 2013 to September 30, 2013).
Written by: Benedict Okeke

Introduction

In the previous quarter, we developed different combinations of acid and alkali pretreatments (2% sulfuric acid, 2% sodium hydroxide) and compared it to concentrated phosphoric acid pretreatments (85% phosphoric acid) followed by either alcohol or acetone. The 85% phosphoric acid followed by acetone resulted in 5.4g/L glucose and 85% phosphoric acid followed by acetone resulted in 4.6 g/L glucose. However, very low concentration of acid and alkali ie., 2% sodium hydroxide followed by 2% sulfuric acid yielded 4.7g/L glucose and the highest xylose yield of 1.5g/L. It was therefore concluded that very low concentration of alkali followed by very low concentration of acid results in high sugar yield, and comparatively, the 85% phosphoric acid treatment gives only 15% higher sugar yield. The use of 1L of 85% phosphoric acid and nearly 5L of acetone for pretreatment of 10% switchgrass seems excessive compared to 1L 2% sodium hydroxide and 1L 2% sulfuric acid for pretreatment of 10% switchgrass for only 15% greater sugar yield. We then concluded that 2% alkali followed by 2% acid is ideal from an economical stand-point and from the perspective of environmental safety. It was also concluded that 0.5% sulfuric acid used singly in switch grass biomass pretreatment resulted in significant conversion to glucose and xylose. Studies in the present report focused on sequential sodium hydroxide and sulfuric acid pretreatments at lower concentrations (0.5% and 1%) and examination of combinations of sodium hydroxide and sulfuric acid pretreatment wastes for production of lignocellulose degrading enzymes and yeast cultivation as well as interaction with the industry on bioethanol equipment. Furthermore, efforts were directed towards data processing for publications as well as preparations for a filling non-provisional patent.

Materials and Methods

Biomass pretreatments

Biomass pretreatment studies focused on sequential dilute alkali-acid pretreatment at lower concentrations (0.5% and 1%). One hundred grams of pulverized switch grass, sieved through 2mm mesh, was suspended in 1L of 0.5% NaOH and subjected to autoclaving at 121^{o}C (15 psi) for 20 min. Total cycle time from start to finish was 1 h. After cooling to room temperature, the NaOH pretreatment liquor was removed by filtering through a piece of a finely woven white canvas tote cloth. The resulting liquor is hereafter referred to "NaOH pretreatment liquor" (NaOH-PTL). Residual liquor was manually squeezed out into a collection tray. Biomass solids were washed twice by re-suspending in 500 mL of 0.5% sulfuric acid each time and removing the acid by filtering and squeezing through a piece of a finely woven white canvas tote cloth. The waste resulting from the two wash cycles is hereafter referred to as "acid wash waste "(H_2SO_4–WL)". For secondary pretreatment, the washed biomass residue was then re-suspended in 500 mL of 0.5% sulfuric acid and subjected to autoclaving and filtration as described above. The resulting waste is hereafter referred to as "acid pretreatment liquor" (H_2SO_4– PTL). Acid was removed by re-suspending and mixing in 500 mL water until the pH of the suspension in water was approximately 4.5. Wash water was then removed by filtering and squeezing through a piece of a finely woven white canvas tote cloth. The wet pretreated biomass was spread out on aluminum foil and allowed to dry at room temperature for 3 days.

Cell-free enzyme production for biomass hydrolysis

One liter of enzyme production medium comprised of the following: KH_2PO_4, 2.0g; $(NH_4)_2SO_4$, 1.2g; $MgSO_4$.$7H_2O$, 0. 5g; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.003g; tween 80, 0.5g; peptone, 1g; yeast extract, 0.5g and 2 ml of trace elements solution (Focht, 1994). Fifty milliliters of the medium in 250 mL Erlenmeyer flask was amended with 0.25g of pulverized waste paper and 0.25g of pulverized switch grass (switch grass culture) and autoclaved at 121^{o}C for 20 min. The sterile media were each inoculated with *Trichoderma* sp. SG2 agar plugs (1.25 cm diameter) obtained from potato dextrose agar plate culture that was incubated at 30^{o}C for three days. Cultures were incubated with orbital shaking (200 rpm) at 30^{o}C for 5 days. Fungal biomass was removed from cultures by centrifugation (8000 rpm, 15 min). The culture supernatant was further cleaned by filtration through a 0.2 micron PES membrane and filtrate (crude enzyme) was used to determine enzyme activities as previously described (Okeke and Lu, 2011).

Saccharification of pretreated biomass using *Trichoderma* SG-2 cell-free crude enzyme

This was examined in 100 mL flasks with airtight caps. Ten milliliters of crude enzyme was placed in each flask and 0.2g of pretreated biomass (2%) was added. The control was a 2% suspension of the biomass in deionized water. Both treatments and controls received 100 µl of lactrol added from a 200 mg/L stock. All flasks were closed tightly to avoid evaporation. The treatments and controls were then incubated at 50°C at 80 rpm in a Thermos Scientific MaxQ 4000 shaker for 72 h and then filtered through 0.45 micron filter before subjecting the filterate to HPLC (Ananda et al 2011). Total reducing sugar was determined by the DNS method (Miller, 1959).

Evaluation of dilute alkali and dilute acid pretreatment wastes (or liquor) utilization for microbial growth and enzyme production

The 0.5% NaOH-PTL (pH 9.2) was combined with 0.5% H_2SO_4– PTL (pH 1.6) at a ratio of 3:2, respectively, with final pH of approximately 4.1. Fifty milliliters of the waste mixture were transferred to 250 mL Erlenmeyer flasks in replicates. In parallel experiments the waste mixture was diluted 50% by combining with distilled water and 50 mL was transferred to 250 mL Erlenmeyer flask in replicates. The media were sterilized by autoclaving at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with two PDA culture plugs (1.25 cm) of *Trichoderma* sp. SG2 culture and incubated at 30^{o}C with orbital shaking (200 rpm) on a shaker incubator. Inoculum was prepared by inoculating the center of potato dextrose agar (PDA) with a piece of PDA culture of *Trichoderma* sp. SG2, incubated at 30^{o}C for 3 days and preserved at 4^{o}C before use. In further experiments enzyme production with a combination of pretreatment wastes-mixture and enzyme production mineral medium was examined. The 0.5% NaOH-PTL (pH 9.2) was combined with 0.5% H_2SO_4-PTL (pH 1.6) as described above Enzyme production medium comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution (Focht, 1994). The waste mixture and enzyme production medium were combined at a ratio of 1:1 and 50 mL was transferred to 250 mL Erlenmeyer flask in replicates. The medium was sterilized by autoclaving at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with two PDA culture plugs (1.25 cm) of *Trichoderma* sp. SG2 culture and incubated at 30^{o}C with orbital shaking (200 rpm).

Validation of the effect of residual acid in combined pretreatment liquor on enzyme activity and saccharification

Un-inoculated controls for enzyme production in combined pretreatment liquors displayed cellulase and xylanase activities. To determine if residual acid in pretreatment liquor contributed to substrate hydrolysis in cellulase and xylanase assays, sulfuric acid treatments of assay mixtures was conducted as given in Tables 4 and 5. Reaction mixtures were incubated at 50^{o}C for 30 min and reducing sugar was determined by the DNS method and used to calculate enzyme activity (Okeke and Lu). Effect of dilute acid addition on saccharification was examined by combining aliquots of 0.5% sulfuric acid with biomass saccharification reaction mixture and controls as shown in Table 6. All flasks were closed tightly to avoid escape of moisture. The treatments and controls were then incubated at 50°C at 80 rpm in a Thermos Scientific MaxQ 4000 shaker for 84 h. Biomass residue was removed by centrifugation (2500 rpm, 10min). Total reducing sugar was determined by the DNS method.

Results

Saccharification of pretreated biomass using *Trichoderma* SG-2 cell-free crude enzyme

We had demonstrated in previous experiments that pretreatments with 2% NaOH followed by 2% H_2SO_4 resulted in significant saccharification of biomass compared to when dilute alkali and dilute acid are used singly. Table 1 presents saccharification of 2% suspension of biomass sequentially pretreated with different concentrations of dilute alkali and acid using cell-free crude enzyme. Total reducing sugar, glucose and xylose concentrations revealed only slight difference between sequential dilute alkali and dilute acid pretreatments at concentrations ranging from 0.5% to 2%. From an economic standpoint, sequential pretreatment of switch grass using 0.5% NaOH and O.5% H_2SO_4 is more cost efficient than sequential pretreatment with 1- 2% alkali and acid.

Evaluation of pretreatment wastes (or liquor) for microbial growth and enzyme production

Pre-treatment liquor was predicted to contain sugars and other nutrients that can support microbial growth. It was also thought that acid and alkaline pretreatment wastes (liquors) from sequential alkali and acid pre-treament could be combined to adjust pH to favor microbial growth and production of different products e.g. enzymes, alcohols including butanol and ethanol. Table 2 presents the results of production of cellulase and xylanase in mixtures of alkali and acid pretreatment liquor (waste). Analysis of reducing sugar in the pretreatment wastes revealed that the acid pretreatment liquors contained the highest amount of sugar (Table 3). In nutrient amended medium cellulase and xylanase production was observed. When compared to boiled enzyme, net cellulase production was low. Xylanase production was, however, higher compared to cellulase production. Results obtained with assays conducted with boiled enzyme indicated that residual acid in the culture supernatant may enhance enzyme activities. Thus, a second set of experiments was set up to evaluate the effects of addition of acid to the assay mixture on cellulase activity (Table 4), xylanase activity (Table 5) and on biomass saccharification (Table 6). Both studies indicated that, in the absence of the enzyme, the low concentration of acid did not have a significant effect on biomass hydrolysis. Based on the drop in pH upon dilute acid addition, it was concluded that it most likely decreased pH to a more favorable range for enzyme activity and biomass saccharification.

Overall, results indicate that alkaline and acid wastes generated from biomass pretreatments can be combined to adjust pH to levels that will favor microbial growth and production of

fermentation products such as enzymes, alcohols and bio-chemicals. Nutrients in the waste can be employed in media formulation for microbial growth and production of fermentation products such as enzymes, alcohols and bio-chemicals. Nonetheless, this requires optimization for better results.

Interaction with the industry for scale up of biomass saccharification, fermentation and distillation

We have interacted with E-fuel Corporation, Los Gatos, CA, about procurement of MicroFueler 300 Series distillation closet (quoted at $29,995), MicroFueler Organic Fuel (feedstock) Tank, 250 gallons capacity including fermentation feature (quoted at $2,995). A model 250 L multi-function (pretreatment, saccharification, pasteurization, fermentation and distillation) bioreactor will be custom made by Zhejiang Dayu Light Industrial Machinary Co. (DAYU), B502-2, Binhai Industrial Park, Economic & Technological Development Zone, Wenzhou, Zhejiang Province, China 325025, tel: +86 577 86806088, (estimated to cost $6,660.00, plus shipping US$950.00). This was designed by making several modifications to the standard "mixing tank" of Dayu Light Industrial Machinary Co. These components will be used to assemble a bioreactor system for scale up using data obtained from this research.

Patent and Manuscript Preparation

We had filed a U.S. Provisional Patent US 61/787,039 titled ***An Efficient Process For Producing Ethanol From A Biomass Feedstock***, with cross-reference to other applications. We invested time in preparation of this non-provisional patent that will be filed soon. We also invested time in manuscript preparation for publication.

Table 1: Saccharification of 2% suspension of switch grass biomass pretreated with different concentrations of dilute alkali and acid using cell-free crude enzyme

Biomass Pretreatments	Reducing Sugar by DNS Method (g/L)	Glucose by HPLC (g/L)	Xylose by HPLC (g/L)
0.5% NaOH primary pretreatment and 0.5% H_2SO_4 secondary pretreatment.	3.744±0.187	3.568± 0.228	1.326±0.065
0.5% NaOH primary pretreatment and 0.5% H_2SO_4 secondary pretreatment control (substrate suspension in water, control).	0.004±0.002	0	0
1% NaOH primary pretreatment and 1% H_2SO_4 secondary pretreatment.	3.863±0.337	3.639 ±0.460	1.181±0.067
1% NaOH primary pretreatment and 1% H_2SO_4 secondary pretreatment control (substrate suspension in water, control).	0.003±0.001	0	0
2% NaOH primary pretreatment and 0.5% H_2SO_4 secondary pretreatment.	3.748±0.631	3.520 ±0.408	1.203±0.058
2% NaOH primary pretreatment and 0.5% H_2SO_4 secondary pretreatment (substrate suspension in water, control).	0.003±0.001	0	1.326±0.065

Table 2: Production of cellulase and xylanase in mixtures of sequential alkali and acid pretreatment liquor

Pre-treatment liquor media	**Cellulase (U/mL/30 min)**	**Xylanase (U/mL/30 min)**
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4–WL (pH 1.7) at a ratio of 1:1 (pH 4.6)	0.71 ± 0.10	3.23± 0.43
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4– PTL (pH 1.6) at a ratio of 3:2 (pH 4.1)	1.47± 0.17	3.61±0.21
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4–WL (pH 1.7) at a ratio of 1:1; and diluted 50% with distilled water	0.91± 0.06	4.08±0.37
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4– PTL (pH 1.6) at a ratio of 3:2; and diluted 50% with distilled water	0.65± 0.06	3.44±0.22
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4– PTL (pH 1.6) at a ratio of 3:2 (pH 4.1); and diluted with equal volume of enzyme production medium	4.39±0.74	31.66±2.19
0.5% NaOH-PTL (pH 9.2) mixed with 0.5% H_2SO_4– PTL (pH 1.6) at a ratio of 3:2 (pH 4.1); and diluted with equal volume of enzyme production medium (**Boiled to inactivate enzyme**)	3.89±0.135	15.11+0.85

Table 3: Analysis of reducing sugar in pretreatment waste.

Treatments	Reducing Sugar (mg/mL)
0.5% NaOH primary pretreatment liquor (NaOH-PTL)	0.893 ± 0.034
0.5% Acid wash waste (H_2SO_4–WL)	0.166 ± 0.036
0.5% Acid secondary pretreatment liquor (H_2SO_4– PTL)	7.772 ± 0.286
1% NaOH primary pretreatment liquor (NaOH-PTL)	1.171 ± 0.111
0.5% Acid wash waste (H_2SO_4–WL)	0.118 ± 0.027
1 % Acid secondary pretreatment liquor (H_2SO_4– PTL)	7.628 ± 0.824

Table 4: Effect of dilute acid addition to reaction mixture on cellulase activity and xylanase activity

Enzyme reaction mixture treatments						
	0.5% H_2SO_4	100 mM CH_3COONa buffer, pH 5.0	Enzyme (Cell-free culture filtrate)	Distilled Water	Final pH	Cellulase (U/mL/30 min)
T1	0.20 ml	0.50 ml	0.25 ml	0.05 ml	3.41	7.26±0.76
T1c	0.20 ml	0.50 ml	0.00 ml	0.30 ml	3.31	0.52±0.17
T2	0.10 ml	0.50 ml	0.25 ml	0.15 ml	3.99	8.76±0.93
T2c	0.10 ml	0.50 ml	0.00ml	0.40 ml	3.90	0.53±0.22
T3	0.05 ml	0.50 ml	0.25 ml	0.20 ml	4.30	7.98±1.44
T3c	0.05 ml	0.50 ml	0.00ml	0.45 ml	4.12	0.81±0.49
TEc	0.00 ml	0.50 ml	0.25 ml	0.25 ml	4.60	7.51±0.64

Table 5: Effect of dilute acid addition to reaction mixture on xylanase activity

Enzyme reaction mixture treatments						
	0.5% H_2SO_4	100 mM CH_3COONa buffer, pH 5.0	Enzyme (cell-free culture filtrate)	Distilled Water	Final pH	Xylanase (U/mL/30 min)
T1	0.20 ml	0.50 ml	0.25 ml	0.05 ml	3.51	56.70 ± 9.41
T1c	0.20 ml	0.50 ml	0.00 ml	0.30 ml	4.12	8.79± 2.04
T2	0.10 ml	0.50 ml	0.25 ml	0.15 ml	4.02	61.55± 11.09
T2c	0.10 ml	0.50 ml	0.00ml	0.40 ml	4.39	7.05± 1.05
T3	0.05 ml	0.50 ml	0.25 ml	0.20 ml	4.2	65.46± 12.59
T3c	0.05 ml	0.50 ml	0.00ml	0.45 ml	4.7	7.28± 1.38
TEc	0.00 ml	0.50 ml	0.25 ml	0.25 ml	3.66	64.08± 7.77

Table 6: Effect of dilute acid addition to reaction mixture on biomass saccharification

Treatments	Enzyme	0.5% H_2SO_4	Water	1% alkali-1% acid pre-treated biomass	Lactrol (200 mg/L)	Reducing sugar (g/L)
T1	10 ml	0ml	1ml	0.2 g	0.1 ml	1.890± 0.038
T1 Control	0ml	0ml	11 ml	0.2 g	0.1 ml	0.013± 0.007
T2	10 ml	1 ml	0ml	0.2g	0.1ml	1.080± 0.142
T2 Control	0ml	1 ml	10ml	0.2g	0.1ml	0.013± 0.010
T3	10ml	0.5 ml	0.5ml	0.2g	0.1ml	2.300± 0.118
T3 Control	0ml	0.5 ml	10.5ml	0.2g	0.1ml	0.007± 0.000

Conclusions and Milestones:

- Total reducing sugar, glucose and xylose concentrations revealed only slight difference between sequential dilute alkali and dilute acid pretreatments at concentrations ranging from 0.5% to 2%. From an economic standpoint, sequential pretreatment of switch grass using 0.5% NaOH and O.5% H_2SO_4 is more cost efficient than sequential pretreatment with 1- 2% alkali and acid.
- Overall, results indicate that alkaline and acid wastes generated from biomass pretreatments can be combined to adjust pH to levels that will favor microbial growth and production of fermentation products such as enzymes, alcohols and bio-chemicals. Nutrients in the waste can be employed in media formulation for microbial growth and production of fermentation products such as enzymes, alcohols and bio-chemicals. Nonetheless, this requires optimization.
- We interacted with the industry for equipment for scale-up process, and plans are in progress to purchase the equipment.
- Filling of non-provisional patent and manuscripts preparation are in progress.

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PART 12: Research Activities of FY-13 (October 1, 2013 to December 30, 2013).
Written by: Benedict Okeke, Professor,

Introduction and Objectives

In previous study , the effects of 10 metal ions: Copper ($CuCl_2$), Barium ($BaCl_2$), Iron ($FeCl_2$), Potassium (KCl), Calcium (CaCl), Magnesium ($MgCl_2$), Manganese ($MnCl_2$) Zinc ($ZnCl_2$), Nickel ($NiCl_2$), Cobalt ($CoCl_2$) and a metal ion chelator, Ethylenediaminetetraacetic acid (EDTA) were examined for their effects on production of cellulolytic and xylanolytic enzymes at initial concentration of 10 mM. Results showed that 10 mM of manganese added as $MnCl_2$ promoted production of cellulase and xylanase in liquid culture compared to control mineral salts medium containing < 0.5 mM Mn. In the same study magnesium added as $MgCl_2$ promoted production of beta-glucosidase and xylosidase in liquid culture compared to control mineral salts medium containing < 0.5 mM Mg. Potassium as KCl amended culture (10 mM) also promoted production of beta xylosidase. When the enzymes produced with metal ions were employed for saccharification of pretreated biomass, glucose release from 2% acid pretreated switch grass was comparable to the control crude enzyme produced without metal ions amendment. Slight improvement of xylose yield from 2% acid pretreated switch grass was observed when the enzyme from K amended culture was employed. Hence further studies were conducted in this quarter to better understand and validate the effects of candidate metal ions on enzyme production. The effects of Mg, Mn and K on production of cellulolytic and xylanolytic enzymes were compared at 10 mM and 20 mM. Direct influence of metal ions and chemicals on cellulolytic and xylanolytic activities was examined by incorporating the metal ions and chemicals in enzyme assay mixtures. Ion Chromatography (IC) profiling of anion nutrients in cellulolytic and xylanolytic cultures was examined. Results were used to study the effects of different dilutions of mineral medium on enzyme production and estimation of the amount of mineral nutrients required for enzyme production.

Methods

Preparation of *Trichoderma* sp. SG2 inoculum

Inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of *Trichoderma* sp. SG2 and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Enzyme production

Enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Fifty mL of the medium was dispensed in to 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switch grass (< 2 mm particle size) and paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 5 days. Enzyme (cell-free culture supernatant) was recovered by centrifugation (8500 rpm, 20 min, 15^{O}C) using an Eppendorf centrifuge.

Enzyme Assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10

discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of oat spelts xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 2% starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. Modified method of Saha et al. 2005 was employed for β-glucosidase activity. The reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath and cooled in ice bath before measuring absorbance at λ_{405}. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside.

Effect of concentrations of Magnesium, Manganese and Potassium on production of cellulase and xylanase

Enzyme production medium comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Forty-nine mL of the medium was dispensed in to 250 mL Erlenmeyer flasks. To the 49 mL basal medium, 0.25 g of pulverized switch grass (< 2 mm) and paper powder (< 2 mm). Replicates of the medium were independently amended with metal ions by adding 1 mL from a 500 mM stock of each metal ion to achieve a final concentration of 10 mM. Two milliliters of the 500 mM stock of each metal ion was added to 48 mL of the medium to achieve a final concentration of 20 mM. Flasks were plugged with foam plug and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture plug (1.25 cm) of *Trichoderma* sp. SG2 culture and incubator at 30^{o}C with orbital shaking (200 rpm). Uninoculated controls were set up to determine the background effect of each metal on the DNS method of sugar determination.

Effect of metal ions and chemicals on cellulolytic and xylanolytic activities

Enzymes were produced using the standard enzyme production medium. Assay mixture for each enzyme was the same as described under enzyme assay except that 20 µl of a 500 mM stock solution of each metal ion or chemical was added to achieve a final concentration of 10 mM. In controls the volume of the enzyme was replaced with water.

Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures

The principal anions in the enzyme production medium are sulfate and phosphate. IC was employed to determine the levels of the anion nutrients in cellulolytic and xylanolytic cultures before and after fermentation. Production of cellulolytic-xylanolytic enzymes was conducted using EPM as described under enzyme production. Inoculum and culture procedures were as described above. Prior to incubation three independent replicates of the cultures were frozen at -80^{o}C and used as control for determination of baseline levels of anion nutrients. Cultures were incubated for 5 days using an orbital incubator, operated at 30^{o}C and 200 rpm. Fungal biomass was removed from all cultures by centrifugation (8500 rpm). Thereafter samples were diluted 10 times, filtered through Phenex-RC 4mm syringe filters AF0-3103-52 (0.45 µ). Five milliliter of each filtered sample was transferred to poly vials and then stoppered with filter caps (20 µm). Samples were analyzed using Dionex ICS 1100 ion chromatograph equipped with conductivity detector. IonPac AS23 analytical column (2 x 250 mm) equipped with IonPac AG23 guard Column (2 x 50 mm) and Dionex anion self-regenerating suppressor

(Dionex ASRS 300 2mm) operated at 7 mA were employed. The eluent was 4.5 mM Na_2CO_3 and 0.8 mM $NaHCO_3$ at a flow rate of 0.25 mL/min. Anion nutrients were estimated using the "Dionex seven anion standard II" (cat. # 057590).

Effect of dilution of culture nutrients on production of cellulolytic and xylanolytic enzymes

Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures revealed that only approximately 50% of anion nutrients (nitrate, phosphate, and sulfate) remained soluble in cell-free culture supernatant suggesting that only about half of the concentration was required for biomass and enzyme production. Hence different dilutions of culture nutrients were examined for enzyme production. The control medium was the complete enzyme production medium (EPM) and comprised of the following per liter: 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$ and 0.003g $FeSO_4.7H_2O$. Two milliliters of Focht mineral elements solution of the following composition per liter: $MnSO_4.H_2O$, 169 mg; $ZnSO_4.7H_2O$, 288 mg; $CuSO_4.5H_2O$, 250 mg; $NiSO_4.6H_2O$, 26 mg; $CoSO_4$, 28 mg; $NaMoO_4$, 24 mg (Focht, 1994), was added. To 50 mL of complete EPM, 0.25g switch grass powder and 0.25 g of paper powder were added. One diluted medium (M2) comprised 50% of complete EPM plus 0.125g switch grass powder and 0.125 g of paper powder added to 50 mL of 50% EPM. Another diluted medium (M3) comprised 50% complete EPM with 0.25g switch grass powder and 0.25 g of paper powder added to 50 mL of 50% EPM. Another diluted medium (M4) comprised 10% of complete EPM plus 0.25g switch grass powder and 0.25 g of paper powder added to 50 mL of 50% EPM.

Patent and Manuscript Preparation

We had filed a U.S. Provisional Patent US 61/787,039 titled *An Efficient Process For Producing Ethanol From A Biomass Feedstock,* with cross-reference to other applications. We invested time in preparation of non-provisional patent. We also invested time in manuscripts preparation.

Interaction with the industry for scale equipment

We are currently interacting with E-fuel corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China for procurement of equipment for scale up studies. Time was spent on design of the process for scale-up studies and sand filter for in-line biomass removal from fermentation broth.

Results

Effect of concentration of Magnesium, Manganese and Potassium on production of cellulase and xylanase

Table 1 presents the effects of different concentrations of candidate metal ions on enzyme production. Cellulase activity was significantly higher when the culture was grown in the presence of Mn compared to the control without Mn. However, similar results were obtained at concentrations of 10 mM and 20 mM. The effect of different concentrations of candidate metal ions on production of xylanase is summarized on Table 2. Mn displayed the highest increase in xylanase activity compared to the control without Mn. At 20 mM of K, xylanase activity was slightly higher than observed in the control without K.

Effect of metal ions and other chemicals on cellulase and xylanase activity

Table 3 shows the effects of metal ions and chemicals on cellulase activity. Addition of Ba, Ca, Co, Fe, K, Mn and Zn displayed stimulation of cellulase activity compared to controls without addition of metal ions. The highest stimulatory effect was observed with Mn, followed by Fe and Ba. Addition of copper to the reaction mixture displayed inhibitory effect. EDTA appears to have little inhibitory effect. Tween 80 did not affect cellulose enzyme activity substantially. Nonetheless, the production medium contained Tween 80 at a level known to support cellulase production. The effects of metal ions and chemicals on xylanase activity is presented on Table 4. Addition of Ba, Ca, Co, Fe, Mn and Zn displayed stimulation of xylanase activity compared to controls without addition of metal ions. The highest stimulatory effect was observed with Mn, followed by Ba and Co. Addition of copper to the reaction mixture displayed only slight inhibitory effect compared to cellulase.

Ion Chromatography profiling of anions nutrients in cellulolytic and xylanolytic cultures and effect of reduction of concentration of mineral nutrients on enzyme production

Table 5 presents ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures. Comparison of the initial concentration of phosphate, sulfate and chloride revealed that over 50% of the initial amounts in culture remained detectable in cell-free culture supernatant. This suggested that half of the mineral nutrient was sufficient for production of the enzymes. Enzyme production with 50% mineral solution revealed that there was no decrease in enzyme activity in cultures although a slight reduction of cellulase activity was observed when 50% substrate (0.125g of switch grass powder and 0.125g paper powder) were added to 50% mineral solution (Table 6, M2). Nonetheless, when the mineral medium was diluted 10%, activities of cellulase, xylanase, β-glucosidase and β-xylosidase significantly dropped (Tables 6-10).

Table 1: Effect of concentration of metal ions on production of cellulase

Metal Ion	Concentration (mM)	Enzyme activity (U/mL/30min)	Standard deviation (U/mL/30min)
Magnesium	10	6.824	1.758
Manganese	10	9.166	3.533
Potassium	10	7.018	1.167
None (Control)	0	6.864	0.599
Magnesium	20	3.005	0.763
Manganese	20	9.659	0.133
Potassium	20	6.997	0.443
None (Control)	0	6.864	0.599

Table 2: Effect of concentration of metal ions on production of xylanase

Metal Ion	Concentration (mM)	Enzyme activity (U/mL/30min)	Standard deviation (U/mL/30min)
Magnesium	10	21.020	4.460
Manganese	10	34.620	6.226
Potassium	10	28.457	5.230
None (Control)	0	28.693	2.899
Magnesium	20	13.776	0.962
Manganese	20	54.531	3.533
Potassium	20	30.561	2.529
None (Control)	0	28.693	2.899

Table 3: Effect of metal ions and other chemicals on cellulase activity

Chemicals (10mM)	Cellulase activity (Units/mL/30min)	SD (Units/mL/30min)	Relative activity (%)	SD (%)
None (Control)	4.780	0.155	100	3.260
Ba	9.808	0.370	205.174	3.774
Ca	6.549	0.740	137.014	11.306
Cu	0.623	0.111	13.033	17.940
Co	6.274	0.347	131.265	5.545
Fe	9.568	0.228	200.172	2.390
EDTA	4.230	0.490	88.500	11.587
K	5.690	0.457	119.046	8.039
Mg	4.156	0.857	86.942	20.635
Mn	13.582	1.762	284.137	12.975
Ni	4.711	0.337	98.562	7.166
Triton-X-100	4.385	0.165	91.734	3.764
Tween 80	4.786	0.567	100.119	11.861
Zn	6.086	0.333	127.331	5.478

Table 4: Effect of metal ions and other chemicals on xylanase activity

Chemicals (10mM)	Cellulase activity (Units/mL/30min)	SD (Units/mL/30min)	Relative activity (%)	SD (%)
None (Control)	21.020	1.443	100	6.864
Ba	30.457	2.684	144.894	8.814
Ca	27.021	1.591	128.54	5.889
Cu	19.577	0.743	93.135	3.796
Co	29.781	2.296	141.680	7.711
Fe	24.507	1.066	116.590	4.352
EDTA	18.569	1.343	88.340	7.235
K	22.486	1.289	106.973	5.736
Mg	20.356	0.592	96.839	2.9104
Mn	39.437	4.075	187.615	10.333
Ni	16.485	1.341	78.424	8.136
Triton-X-100	24.020	0.915	114.274	3.809
Tween 80	24.227	1.306	115.255	5.391
Zn	24.357	2.087	115.875	8.568

Table 5: Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures

Anion Nutrient	Initial Concentration (mg/L)		Final Concentration (mg/L)		% Detectable in Soluble Fraction
	Concentration	SD	Concentration	SD	
Phosphate	525.299	2.824	297.624	28.907	56.66
Sulfate	1403.517	16.634	797.682	112.470	56.83
Chloride	99.023	8.0564	66.206	2.802	66.86

Table 6: Effect of dilution medium nutrients on production of cellulase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	Cellulase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	10.034	0.448
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	9.497	0.327
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	11.150	0.567
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	5.612	0.063

The mineral solution comprised 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution (Focht, 1994). The Fotch mineral element solution comprised $MnSO_4.H_2O$, 169 mg; $ZnSO_4.7H_2O$, 288 mg; $CuSO_4.5H_2O$, 250 mg; $NiSO_4.6H_2O$, 26 mg; $CoSO_4$, 28 mg and $NaMoO_4$, 24 mg per liter.

Table 7: Effect of dilution medium nutrients on production of xylanase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	Xylanase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	41.137	2.616
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	41.507	1.114
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	41.803	1.092
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	39.155	2.486

Table 8: Effect of dilution medium nutrients on production of β-glucosidase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	β-glucosidase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	21.035	0.191
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	15.647	4.839
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	28.885	1.828
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	7.335	0.999

Table 9: Effect of dilution medium nutrients on production of β-xylosidase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	β-xylosidase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	13.910	2.348
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	9.872	0.390
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	15.297	2.356
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	4.535	0.555

Conclusions

Manganese was validated to improve cellulase and xylanase production in *Trichoderma* sp SG2. Addition of Ba, Ca, Co, Fe, K, Mn and Zn to enzyme assay mixture displayed stimulation of enzyme activity compared to assays without addition of metal ions. Results indicate that metal ions can be added to biomass saccharification reaction mixture to improve sugar yield. Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures revealed that over 50% of the initial anion nutrients remained detectable in cell-free culture supernatant and suggested that half of the mineral nutrients was sufficient for production of cellulolytic and xylanolytic enzymes of *Trichoderma* sp. SG2. Enzyme production with 50% mineral solution revealed that there was no decrease of enzyme activity in cultures.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, WI, pp 407–426.
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PART 12: Research Activities of FY-14 (October 1, 2013 to December 30, 2013).
Written by: Benedict Okeke

Introduction and Objectives

In previous study , the effects of 10 metal ions: Copper ($CuCl_2$), Barium ($BaCl_2$), Iron ($FeCl_2$), Potassium (KCl), Calcium (CaCl), Magnesium ($MgCl_2$), Manganese ($MnCl_2$) Zinc ($ZnCl_2$), Nickel ($NiCl_2$), Cobalt ($CoCl_2$) and a metal ion chelator, Ethylenediaminetetraacetic acid (EDTA) were examined for their effects on production of cellulolytic and xylanolytic enzymes at initial concentration of 10 mM. Results showed that 10 mM of manganese added as $MnCl_2$ promoted production of cellulase and xylanase in liquid culture compared to control mineral salts medium containing < 0.5 mM Mn. In the same study magnesium added as $MgCl_2$ promoted production of beta-glucosidase and xylosidase in liquid culture compared to control mineral salts medium containing < 0.5 mM Mg. Potassium as KCl amended culture (10 mM) also promoted production of beta xylosidase. When the enzymes produced with metal ions were employed for saccharification of pretreated biomass, glucose release from 2% acid pretreated switch grass was comparable to the control crude enzyme produced without metal ions amendment. Slight improvement of xylose yield from 2% acid pretreated switch grass was observed when the enzyme from K amended culture was employed. Hence further studies were conducted in this quarter to better understand and validate the effects of candidate metal ions on enzyme production. The effects of Mg, Mn and K on production of cellulolytic and xylanolytic enzymes were compared at 10 mM and 20 mM. Direct influence of metal ions and chemicals on cellulolytic and xylanolytic activities was examined by incorporating the metal ions and chemicals in enzyme assay mixtures. Ion Chromatography (IC) profiling of anion nutrients in cellulolytic and xylanolytic cultures was examined. Results were used to study the effects of different dilutions of mineral medium on enzyme production and estimation of the amount of mineral nutrients required for enzyme production.

Methods

Preparation of *Trichoderma* sp. SG2 inoculum

Inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of *Trichoderma* sp. SG2 and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Enzyme production

Enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Fifty mL of the medium was dispensed in to 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switch grass (< 2 mm particle size) and paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 5 days. Enzyme (cell-free culture supernatant) was recovered by centrifugation (8500 rpm, 20 min, 15^{O}C) using an Eppendorf centrifuge.

Enzyme Assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of oat spelts xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 2% starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. Modified method of Saha et al. 2005 was employed for β-glucosidase activity. The reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath and cooled in ice bath before measuring absorbance at λ_{405}. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside.

Effect of concentrations of Magnesium, Manganese and Potassium on production of cellulase and xylanase

Enzyme production medium comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Forty-nine mL of the medium was dispensed in to 250 mL Erlenmeyer flasks. To the 49 mL basal medium, 0.25 g of pulverized switch grass (< 2 mm) and paper powder (< 2 mm). Replicates of the medium were independently amended with metal ions by adding 1 mL from a 500 mM stock of each metal ion to achieve a final concentration of 10 mM. Two milliliters of the 500 mM stock of each metal ion was added to 48 mL of the medium to achieve a final concentration of 20 mM. Flasks were plugged with foam plug and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture plug (1.25 cm) of *Trichoderma* sp. SG2 culture and incubator at 30^{o}C with orbital shaking (200 rpm). Uninoculated controls were set up to determine the background effect of each metal on the DNS method of sugar determination.

Effect of metal ions and chemicals on cellulolytic and xylanolytic activities

Enzymes were produced using the standard enzyme production medium. Assay mixture for each enzyme was the same as described under enzyme assay except that 20 µl of a 500 mM stock solution of each metal ion or chemical was added to achieve a final concentration of 10 mM. In controls the volume of the enzyme was replaced with water.

Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures

The principal anions in the enzyme production medium are sulfate and phosphate. IC was employed to determine the levels of the anion nutrients in cellulolytic and xylanolytic cultures before and after fermentation. Production of cellulolytic-xylanolytic enzymes was conducted using EPM as described under enzyme production. Inoculum and culture procedures were as described above. Prior to incubation three independent replicates of the cultures were frozen at -80^{o}C and used as control for determination of baseline levels of anion nutrients. Cultures were incubated for 5 days using an orbital incubator, operated at 30^{o}C and 200 rpm. Fungal biomass was removed from all cultures by centrifugation (8500 rpm). Thereafter samples were diluted 10 times, filtered through Phenex-RC 4mm syringe filters AF0-3103-52 (0.45 µ). Five milliliter of each filtered sample was transferred to poly vials and then stoppered with

filter caps (20 μm). Samples were analyzed using Dionex ICS 1100 ion chromatograph equipped with conductivity detector. IonPac AS23 analytical column (2 x 250 mm) equipped with IonPac AG23 guard Column (2 x 50 mm) and Dionex anion self-regenerating suppressor (Dionex ASRS 300 2mm) operated at 7 mA were employed. The eluent was 4.5 mM Na_2CO_3 and 0.8 mM $NaHCO_3$ at a flow rate of 0.25 mL/min. Anion nutrients were estimated using the "Dionex seven anion standard II" (cat. # 057590).

Effect of dilution of culture nutrients on production of cellulolytic and xylanolytic enzymes

Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures revealed that only approximately 50% of anion nutrients (nitrate, phosphate, and sulfate) remained soluble in cell-free culture supernatant suggesting that only about half of the concentration was required for biomass and enzyme production. Hence different dilutions of culture nutrients were examined for enzyme production. The control medium was the complete enzyme production medium (EPM) and comprised of the following per liter: 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$ and 0.003g $FeSO_4.7H_2O$. Two milliliters of Focht mineral elements solution of the following composition per liter: $MnSO_4.H_2O$, 169 mg; $ZnSO_4.7H_2O$, 288 mg; $CuSO_4.5H_2O$, 250 mg; $NiSO_4.6H_2O$, 26 mg; $CoSO_4$, 28 mg; $NaMoO_4$, 24 mg (Focht, 1994), was added. To 50 mL of complete EPM, 0.25g switch grass powder and 0.25 g of paper powder were added. One diluted medium (M2) comprised 50% of complete EPM plus 0.125g switch grass powder and 0.125 g of paper powder added to 50 mL of 50% EPM. Another diluted medium (M3) comprised 50% complete EPM with 0.25g switch grass powder and 0.25 g of paper powder added to 50 mL of 50% EPM. Another diluted medium (M4) comprised 10% of complete EPM plus 0.25g switch grass powder and 0.25 g of paper powder added to 50 mL of 50% EPM.

Patent and Manuscript Preparation

We had filed a U.S. Provisional Patent US 61/787,039 titled ***An Efficient Process For Producing Ethanol From A Biomass Feedstock***, with cross-reference to other applications. We invested time in preparation of non-provisional patent. We also invested time in manuscripts preparation.

Interaction with the industry for scale equipment

We are currently interacting with E-fuel corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China for procurement of equipment for scale up studies. Time was spent on design of the process for scale-up studies and sand filter for in-line biomass removal from fermentation broth.

Results

Effect of concentration of Magnesium, Manganese and Potassium on production of cellulase and xylanase

Table 1 presents the effects of different concentrations of candidate metal ions on enzyme production. Cellulase activity was significantly higher when the culture was grown in the presence of Mn compared to the control without Mn. However, similar results were obtained at concentrations of 10 mM and 20 mM. The effect of different concentrations of candidate metal ions on production of xylanase is summarized on Table 2. Mn displayed the highest increase in xylanase activity compared to the control without Mn. At 20 mM of K, xylanase activity was slightly higher than observed in the control without K.

Effect of metal ions and other chemicals on cellulase and xylanase activity

Table 3 shows the effects of metal ions and chemicals on cellulase activity. Addition of Ba, Ca, Co, Fe, K, Mn and Zn displayed stimulation of cellulase activity compared to controls without addition of metal ions. The highest stimulatory effect was observed with Mn, followed by Fe and Ba. Addition of copper to the reaction mixture displayed inhibitory effect. EDTA appears to have little inhibitory effect. Tween 80 did not affect cellulose enzyme activity substantially. Nonetheless, the production medium contained Tween 80 at a level known to support cellulase production. The effects of metal ions and chemicals on xylanase activity is presented on Table 4. Addition of Ba, Ca, Co, Fe, Mn and Zn displayed stimulation of xylanase activity compared to controls without addition of metal ions. The highest stimulatory effect was observed with Mn, followed by Ba and Co. Addition of copper to the reaction mixture displayed only slight inhibitory effect compared to cellulase.

Ion Chromatography profiling of anions nutrients in cellulolytic and xylanolytic cultures and effect of reduction of concentration of mineral nutrients on enzyme production

Table 5 presents ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures. Comparison of the initial concentration of phosphate, sulfate and chloride revealed that over 50% of the initial amounts in culture remained detectable in cell-free culture supernatant. This suggested that half of the mineral nutrient was sufficient for production of the enzymes. Enzyme production with 50% mineral solution revealed that there was no decrease in enzyme activity in cultures although a slight reduction of cellulase activity was observed when 50% substrate (0.125g of switch grass powder and 0.125g paper powder) were added to 50% mineral solution (Table 6, M2). Nonetheless, when the mineral medium was diluted 10%, activities of cellulase, xylanase, β-glucosidase and β-xylosidase significantly dropped (Tables 6-10).

Table 1: Effect of concentration of metal ions on production of cellulase

Metal Ion	Concentration (mM)	Enzyme activity (U/mL/30min)	Standard deviation (U/mL/30min)
Magnesium	10	6.824	1.758
Manganese	10	9.166	3.533
Potassium	10	7.018	1.167
None (Control)	0	6.864	0.599
Magnesium	20	3.005	0.763
Manganese	20	9.659	0.133
Potassium	20	6.997	0.443
None (Control)	0	6.864	0.599

Table 2: Effect of concentration of metal ions on production of xylanase

Metal Ion	Concentration (mM)	Enzyme activity (U/mL/30min)	Standard deviation (U/mL/30min)
Magnesium	10	21.020	4.460
Manganese	10	34.620	6.226
Potassium	10	28.457	5.230
None (Control)	0	28.693	2.899
Magnesium	20	13.776	0.962
Manganese	20	54.531	3.533
Potassium	20	30.561	2.529
None (Control)	0	28.693	2.899

Table 3: Effect of metal ions and other chemicals on cellulase activity

Chemicals (10mM)	Cellulase activity (Units/mL/30min)	SD (Units/mL/30min)	Relative activity (%)	SD (%)
None (Control)	4.780	0.155	100	3.260
Ba	9.808	0.370	205.174	3.774
Ca	6.549	0.740	137.014	11.306
Cu	0.623	0.111	13.033	17.940
Co	6.274	0.347	131.265	5.545
Fe	9.568	0.228	200.172	2.390
EDTA	4.230	0.490	88.500	11.587
K	5.690	0.457	119.046	8.039
Mg	4.156	0.857	86.942	20.635
Mn	13.582	1.762	284.137	12.975
Ni	4.711	0.337	98.562	7.166
Triton-X-100	4.385	0.165	91.734	3.764
Tween 80	4.786	0.567	100.119	11.861
Zn	6.086	0.333	127.331	5.478

Table 4: Effect of metal ions and other chemicals on xylanase activity

Chemicals (10mM)	Cellulase activity (Units/mL/30min)	SD (Units/mL/30min)	Relative activity (%)	SD (%)
None (Control)	21.020	1.443	100	6.864
Ba	30.457	2.684	144.894	8.814
Ca	27.021	1.591	128.54	5.889
Cu	19.577	0.743	93.135	3.796
Co	29.781	2.296	141.680	7.711
Fe	24.507	1.066	116.590	4.352
EDTA	18.569	1.343	88.340	7.235
K	22.486	1.289	106.973	5.736
Mg	20.356	0.592	96.839	2.9104
Mn	39.437	4.075	187.615	10.333
Ni	16.485	1.341	78.424	8.136
Triton-X-100	24.020	0.915	114.274	3.809
Tween 80	24.227	1.306	115.255	5.391
Zn	24.357	2.087	115.875	8.568

Table 5: Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures.

Anion Nutrient	Initial Concentration (mg/L)		Final Concentration (mg/L)		% Detectable in Soluble Fraction
	Concentration	SD	Concentration	SD	
Phosphate	525.299	2.824	297.624	28.907	56.66
Sulfate	1403.517	16.634	797.682	112.470	56.83
Chloride	99.023	8.0564	66.206	2.802	66.86

Table 6: Effect of dilution medium nutrients on production of cellulase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	Cellulase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	10.034	0.448
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	9.497	0.327
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	11.150	0.567
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	5.612	0.063

The mineral solution comprised 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$, and 2ml of Fotch mineral element solution (Focht, 1994). The Fotch mineral element solution comprised $MnSO_4.H_2O$, 169 mg; $ZnSO_4.7H_2O$, 288 mg; $CuSO_4.5H_2O$, 250 mg; $NiSO_4.6H_2O$, 26 mg; $CoSO_4$, 28 mg and $NaMoO_4$, 24 mg per liter.

Table 7: Effect of dilution medium nutrients on production of xylanase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	Xylanase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	41.137	2.616
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	41.507	1.114
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	41.803	1.092
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	39.155	2.486

Table 8: Effect of dilution medium nutrients on production of β-glucosidase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	β-glucosidase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	21.035	0.191
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	15.647	4.839
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	28.885	1.828
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	7.335	0.999

Table 9: Effect of dilution medium nutrients on production of β-xylosidase by *Trichoderma* sp. SG2

Media	Mineral solution	Tween-80	Peptone	Yeast extract	Switch grass powder	Paper powder	β-xylosidase U/mL/30min	SD
M1	100%	0.5g/L	1.0g/L	0.5g/L	0.25g/50mL	0.25g/50mL	13.910	2.348
M2	50%	0.5g/L	0.5g/L	0.25g/L	0.125/50mL	0.125g/50mL	9.872	0.390
M3	50%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	15.297	2.356
M4	10%	0.5g/L	1.0g/L	0.5g/L	0.25/50mL	0.25g/50mL	4.535	0.555

Conclusions

Manganese was validated to improve cellulase and xylanase production in *Trichoderma* sp SG2. Addition of Ba, Ca, Co, Fe, K, Mn and Zn to enzyme assay mixture displayed stimulation of enzyme activity compared to assays without addition of metal ions. Results indicate that metal ions can be added to biomass saccharification reaction mixture to improve sugar yield. Ion chromatography (IC) profiling of anions nutrients in cellulolytic and xylanolytic cultures revealed that over 50% of the initial anion nutrients remained detectable in cell-free culture supernatant and suggested that half of the mineral nutrients was sufficient for production of cellulolytic and xylanolytic enzymes of *Trichoderma* sp. SG2. Enzyme production with 50% mineral solution revealed that there was no decrease of enzyme activity in cultures.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, WI, pp 407–426.
2. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry* 31: 426-428.
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4. Saha, B. C., Iten, L. B., Cotta, M. A., & Wu, Y. V. (2005). Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol.Biotechnology Progress, 21, 816–822.

PART 12: Research Activities of FY-13 (January 1, 2014 to March 30, 2014).
Written by: Benedict Okeke, Professor

Introduction and Objectives

In previous quarter, Mn was validated to improve cellulase and xylanase production in *Trichoderma* sp. SG2. The effects of metal ions on promotion of cellulase and xylanase activities were evaluated. Addition of Ba, Ca, Co, Fe, K, Mn and Zn to enzyme assay mixture displayed stimulation of enzyme activity compared to assays without addition of metal ions. Results indicated that metal ions can be added to biomass saccharification reaction mixture to improve sugar yield. Thus, further studies were carried out to determine the influence of metal ions biomass saccharification by *Trichoderma* sp. SG2 cellulolytic and xylanolytic enzymes complex. Levels of enzymes available for biomass saccharifcation in crude cultures were estimated by assay of different dilutions of the culture filtrates. Time was spent on data processing, manuscript preparation and filling of non-provisional US patent based on the results of the study.

Methods

Preparation of *Trichoderma* sp. SG2 inoculum

Inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of *Trichoderma* sp. SG2 and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Enzyme production

Enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Fifty milliliters of the medium was dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switch grass (< 2 mm particle size) and paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 5 days. Enzyme (cell-free culture supernatant) was recovered by centrifugation (8500 rpm, 20 min, 15^{O}C) using an Eppendorf centrifuge.

Enzyme Assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 2% starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. Enzyme activity (µmoles of glucose) was read from a glucose standard curve.

Effect of metal ions on biomass saccharification

Switchgrass (*Panicum virgatum*) biomass sequentially pre-treated with 1% NaOH with autoclaving at 121^{o}C for 20 min and 1% sulfuric acid with autoclaving at 121^{o}C for 20 min (FY13_Fourth_Quarter_Report), was used. In one experiment, the reaction mixture comprised of 0.2g of pretreated switchgrass, 10.0 mL of enzyme (cell-free culture supernatant) 200µL of Ba, Ca and Mn stock solutions (500 mM), and 100µL of 200mg/L lactrol in 125 mL Erlenmeyer flask. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0mL of distilled water. In another experiment in which combinations of the metal ions (Ba, Ca and Mn) was examined, the reaction mixture comprised of 0.1g of pretreated switchgrass, 5.0 mL of enzyme (cell-free culture supernatant) 100µL of metal ion stock, and 50µL of 200 mg/L lactrol in 15 mL centrifuge tube. The control was the same as the reaction mixture except that the crude enzyme was replaced with 5.0mL of distilled water. The reactions were incubated at 50^{o}C in an orbital incubator (80 rpm) for 24 hours. When the reaction was carried out in 15 ml centrifuge tubes, the tubes were placed horizontally on the shaker incubator. Post incubation, 100 µL of digest was diluted in 900 µl of deionized water. Sugar concentration was measured in 1 mL of the dilute digest using the DNS method.

Estimation of Levels of Enzymes in Culture Filtrate for Biomass Saccharification

Four fungal isolates (*Trichoderma* SG2*, Trichoderma* SG4, *Penicillium sp* FS22A, *Trichoderma* FS5A**)** were grown as described under enzyme production. Enzyme (cell-free culture filtrate) was diluted two times (50%), four times (25%) and ten times (10%) using 50 mM sodium acetate buffer, pH 5.0. The different dilutions of the enzyme were then employed to determine activities as previously described.

Patent and Manuscript Preparation

We spent time on filling of a non-provisional U.S. Patent (Application number 14205779) titled An Efficient Process for Producing Saccharides and Ethanol from a Biomass Feedstock, Cross Reference to Related Applications. We also invested time in manuscripts preparation.

Interaction with the industry for scale-up equipment

We interacted with E-fuel Corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China for procurement of equipment for scale up studies.

Results

Effect of different concentrations of candidate metal ions and combination of metal ions on biomass saccharification

The effects of different concentrations of candidate metal ions on biomass saccharification are presented on Table 1. Ba was most effective in promotion of biomass saccharification at 1-5 mM. Ca was most effective at 5 mM. Whereas Mn was most effective at 1 mM. The highest promotion of biomass hydrolysis (1.93 times) was observed with 1mM Mn followed by Ca which displayed 1.89 times increase in biomass hydrolysis at 5 mM. The metal ions were evaluated for potential synergistic effect in promotion of biomass hydrolysis (Table 2). Combinations of the three metal ions did not result to increase in biomass hydrolysis. Nonetheless, results obtained with combination of Ba/Mn at 1 mM, Ba/Ca at 5 mM and Ca/Mn at 1 mM, further support significant promotion of biomass hydrolysis by the metal ions. Results indicate potential application of each of the three metal ions at concentrations ranging from 1-5 mM in production of saccharides from biomass.

Levels of Enzymes in Culture Filtrate for Biomass Saccharification

Tables 3-6 present estimation of total cellulase, xylanase and amylase activities in cell-free culture supernatant of *Trichoderma* SG2, *Trichoderma* SG4, *Penicillium* FS22A and *Trichoderma* FS5A, respectively. Dilution of the enzymes before assay revealed that more enzymes are present in the cell-free culture supernatant than required to contact with/and convert the substrate to fermentable sugars. At 10% enzyme dilution, results indicate that cellulase, xylanase and amylase are about 5, 8 and 7 orders of magnitude higher than observed in assays conducted using the cell-free culture without dilution of *Trichoderma* SG2. Thus, less enzyme can be used or more substrates can be added to the reaction to maximize saccharide yield.

Table 1: Effect of metal ions on biomass saccharification

Metal Ions	Concentration	Reducing sugar (mg/mL)	SD (mg/mL)	Relative effect (%)	Relative effect SD (%)
None	0 mM	3.985	0.145	100	3.641
Barium	1 mM	6.053	0.467	151.886	11.733
	5 mM	5.964	0.309	149.649	7.776
	10 mM	5.250	0.256	131.747	6.429
Calcium	1 mM	6.075	0.276	152.454	6.936
	5 mM	7.526	0.292	188.846	7.328
	10 mM	6.862	0.426	172.1832	10.690
Manganese	1 mM	7.701	0.195	193.244	4.917
	5 mM	6.298	0.030	158.041	0.758
	10 mM	ND	ND	ND	ND

None: no addition to the reaction. Enzyme contains residual metal ions from production medium

ND: not determined conclusively.

Table 2: Effect of combination of metal ions on biomass saccharification

Metal Ion Combinations	Concentration	Reducing sugar (mg/mL)	SD (mg/mL)	Relative effect (%)	Relative effect SD (%)
None	0 mM	4.587	0.243	100	5.307
Barium/ Manganese	1 mM	7.146	0.244	155.786	5.338
	5 mM	5.289	0.921	115.310	20.079
	10 mM	ND	ND	ND	ND
Barium/ Calcium	1 mM	5.11	0.292	111.418	6.372
	5 mM	6.690	0.191	145.849	4.170
	10 mM	5.475	0.108	119.362	2.365
Calcium /Manganese	1 mM	7.116	0.281	155.121	6.134
	5 mM	5.977	0.820	130.297	17.886
	10 mM	6.843	0.426	149.182	9.294

None: no addition to the reaction. Enzyme contains residual metal ions from production medium.

ND: not determined conclusively.

Table 3: Estimation of total cellulase, xylanase and amylase activities in cell-free culture supernatant of *Trichoderma* SG2

Enzymes	Concentration	Activity[a] (Units/ml/30min)	SD	Relative Activity (%)	Magnitude[b]
Cellulase	100%	9.837	1.121	100	1
	50%	16.863	1.3144	171.416	1.714
	25%	26.129	2.473	265.594	2.655
	10%	52.540	1.049	534.060	5.340
Xylanase	100%	48.017	2.529	100	1
	50%	95.294	6.317	198.457	1.984
	25%	175.108	14.482	364.678	3.646
	10%	405.648	23.989	844.798	8.447
Amylase	100%	25.732	0.542	100	1
	50%	45.523	0.419	176.910	1.769
	25%	84.488	0.501	328.334	3.283
	10%	195.882	0.971	761.227	7.612

[a]Enzyme activities were multiplied with the dilution factor.
[b]Enzyme activity relative to the activity obtained with 100% enzyme (undiluted cell-free culture supernatant).

Table 4: Estimation of total cellulase, xylanase and amylase activities in cell-free culture supernatant of *Trichoderma* SG4

Enzymes	Concentration	Activity[a] (Units/ml/30min)	SD	Relative Activity (%)	Magnitude[b]
Cellulase	100%	6.218	0.305	100	1
	50%	8.067	0.654	129.722	1.297
	25%	10.597	0.277	170.405	1.704
	10%	15.474	2.008	248.831	2.488
Xylanase	100%	22.082	2.316	100	1
	50%	55.424	4.917	250.987	2.509
	25%	101.071	4.640	457.695	4.576
	10%	213.603	24.240	967.294	9.672
Amylase	100%	23.283	0.407	100	1
	50%	38.530	0.787	165.482	1.654
	25%	48.690	1.313	209.117	2.091
	10%	56.540	1.864	242.833	2.428

[a]Enzyme activities were multiplied with the dilution factor.
[b]Enzyme activity relative to the activity obtained with 100% enzyme (undiluted cell-free culture supernatant).

Table 5: Estimation of total cellulase, xylanase and amylase activities in cell-free culture supernatant of *Penicillium* FS22A

Enzymes	Concentration	Activity[a] (Units/ml/30min)	SD	Relative Activity (%)	Magnitude[b]
Cellulase	100%	7.265	0.466	100	1
	50%	10.289	1.175	141.627	1.416
	25%	15.004	0.737	206.530	2.065
	10%	20.937	0.932	288.190	2.881
Xylanase	100%	20.897	1.212	100	1
	50%	43.128	4.123	206.380	2.063
	25%	84.997	2.459	406.734	4.067
	10%	166.751	7.481	797.954	7.979
Amylase	100%	2.931	0.093	100	1
	50%	3.752	0.444	127.993	1.279
	25%	3.949	0.171	134.714	1.347
	10%	5.8	1.710	197.827	1.978

[a]Enzyme activities were multiplied with the dilution factor.
[b]Enzyme activity relative to the activity obtained with 100% enzyme (undiluted cell-free culture supernatant).

Table 6: Effect of dilution of enzymes (cell-free culture supernatant) on cellulase, xylanase and amylase in cell-free culture supernatant of *Trichoderma* FS5A

Enzymes	Concentration	Activity[a] (Units/ml/30min)	SD	Relative Activity (%)	Magnitude[b]
Cellulase	100%	5.450	0.488	100	1
	50%	7.955	0.367	145.975	1.459
	25%	9.763	0.632	179.144	1.791
	10%	13.918	0.649	255.376	2.553
Xylanase	100%	21.564	1.625	100	1
	50%	38.720	1.855	179.561	1.795
	25%	69.737	2.609	323.397	3.233
	10%	162.492	2.211	753.533	7.535
Amylase	100%	9.135	0.375	100	1
	50%	10.974	0.666	120.132	1.201
	25%	12.838	0.705	140.533	1.405
	10%	17.651	0.604	193.221	1.932

[a]Enzyme activities were multiplied with the dilution factor.
[b]Enzyme activity relative to the activity obtained with 100% enzyme (undiluted cell-free culture supernatant).

Conclusions

Ba, Ca and Mn substantially promoted biomass hydrolysis at orders of magnitude ranging from 1.5 to 1.9 times at 1-5 mM. The highest promotion of biomass hydrolysis (1.93 times) was observed with 1mM Mn followed by Ca which displayed 1.89 times increase in biomass hydrolysis at 5 mM. Synergistic effect in promotion of biomass hydrolysis was not observed when the three metal ions were examined in combinations. Dilution of the enzymes before assay revealed that more enzymes are present in the cell-free culture supernatant than required to contact with/and convert the substrate to saccharides. Overall, results suggest potential application of *Trichoderma* SG2 for production of saccharides from biomass with Ba, Ca or Mn supplementation. Interestingly, *Trichoderma* SG2 co-produces cellulolytic-xylanolytic-amylolytic enzymes. This is an indication that starchy carbohydrate wastes can be added as adjuncts for higher yield of fermentable saccharides.

References

1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, WI, pp 407–426.
2. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry* 31: 426-428.
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PART 13: Research Activities of FY-13 (April 1, 2014 to June 30, 2014).
Written by: Benedict Okeke, Professor, bokeke@aum.edu, 3342443508

Introduction and objectives

In previous report, Ba, Ca and Mn substantially promoted biomass hydrolysis at orders of magnitude ranging from 1.5 to 1.9 times at 1-5 mM. The highest promotion of biomass hydrolysis (1.93 times) was observed with Mn followed by Ca which displayed 1.89 times increase in biomass hydrolysis at 5 mM. Synergistic effect in promotion of biomass hydrolysis was not observed when the three metal ions were examined in combinations. Dilution of the enzymes before assay revealed that more enzymes are present in the cell-free culture supernatant than required to contact with/and convert the substrate to saccharides. Results suggest potential application of *Trichoderma* SG2 for production of saccharides from biomass with Ba, Ca or Mn supplementation.

In this report, HPLC analysis was employed to determine the amounts of glucose and xylose released in saccharification reaction mixtures amended with Ba, Ca and Mn and to validate metal ion stimulated biomass saccharification by cellulolytic and xylanolytic enzymes of *Trichoderma* sp. SG2. Time was spent on data processing, manuscript preparation and conference presentations. Effort was also spent on making arrangements for construction of equipment for scale up studies from the industry: E-fuel Corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China.

Methods

Production of enzymes

Enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Fifty milliliters of the medium was dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 5 days. Enzyme (cell-free culture supernatant) was recovered by centrifugation (8500 rpm, 20 min, 15^{O}C) using an Eppendorf centrifuge. *Trichoderma* s*p.* SG2 inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of *Trichoderma* sp. SG2 and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Enzyme assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 2% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. Enzyme activity (μmoles of glucose) was read from a glucose standard curve.

HPLC analysis of effect of metal ions on glucose and xylose release from biomass saccharification

Switchgrass biomass was sequentially pre-treated with 1% NaOH with autoclaving at 121^{o}C for 20 min and 1% sulfuric acid with autoclaving at 121^{o}C for 20 min (FY13_Fourth_Quarter_Report). Saccharification reaction mixture comprised of 0.2g of pretreated switchgrass, 10.0 mL of enzyme (cell-free culture supernatant), 100µL of 200mg/L lactrol and either 20µL, 100 µL or 200µL of 500 mM stock solutions of Ba, Ca and Mn in 125 mL Erlenmeyer flasks. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0mL of distilled water. The reactions were incubated at 50^{o}C in an orbital incubator (80 rpm) for 24 hours. Post incubation, 100 µL of digest was diluted in 900 µl of deionized water. Sugars in each sample were quantified using HPLC according to a published method (Nanjundaswamy et al. 2011). Briefly, Shimadzu HPLC equipped with RID detector and Lab solution software was used for determining levels of glucose. For separation, Phenomenex Rezex-ROA organic acid column cross-linked with 8% hydrogen resin was used. Column oven temperature was operated at 82°C. HPLC-grade water was used as the mobile phase at a flow rate of 0.6 ml/min.

Effect of pH on biomass saccharification

The reaction mixture comprised of 0.2 g of 1% alkali-1% acid pretreated switchgrass biomass, 10.0 mL of enzyme (cell-free culture supernatant), and predetermined aliquots of 0.5% sulfuric acid (w/v) were independently added to adjust the pH to 6.0, 5.5, 5.0, 4.5 and 4.0 as shown in Table 1. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0 mL of distilled water. The reactions were incubated at 50^{o}C in an orbital incubator (80 rpm) for 24 hours. Post incubation, 100 µL of digest was diluted in 900 µl of deionized water and reducing sugar was determined by the DNS method (Miller, 1959).

Table 1: pH adjustment procedure for biomass saccharification.

Enzyme (mL)	Biomass (g)	0.5% H_2SO_4 w/v, (µL)	pH
10 mL	0.2	0	6.27
10 mL	0.2	100	6.00
10 mL	0.2	160	5.50
10 mL	0.2	195	5.00
10 mL	0.2	275	4.50
10 mL	0.2	320	4.00

Results

Glucose and xylose release from biomass saccharification reactions amended with metal ions

Figure 1 presents HPLC analysis of glucose release from switchgrass biomass in reactions amended with Ba, Ca and Mn at different concentrations. At 1-5 mM the metal ions displayed significant stimulation of glucose release from switchgrass biomass. Xylose release from switchgrass biomass in reactions amended with Ba, Ca and Mn at different concentrations is shown in Figure 2. Significant promotion of xylose release was recorded with the three metal ions from 1-5 mM. Ca and Mn displayed higher stimulation of glucose and xylose release from switchgrass biomass, than Ba. Figure 3 presents total glucose and xylose produced from 2% pretreated switchgrass in reactions amended with Ba, Ca and Mn at different concentrations. An analysis of the relative effects of the metal ions is presented in Table 2. Promotion of glucose release by calcium and manganese were about 2.74 and 2.40 orders of magnitude, respectively at 5 mM. Whereas promotion of xylose release by calcium and manganese were about 4.25 and 4.51 orders of magnitude, respectively at 5 mM. Total glucose and xylose yield at 5 mM increased by 3.05 and 2.79 orders of magnitude, respectively.

Effect of pH on biomass saccharification by *Trichoderma* SG2 enzyme

Table 3 presents the effects of pH on biomass saccharification by *Trichoderma* SG2 enzyme. Release of total reducing sugar from biomass was maximal at initial pH 5.0 Similar results were recorded at initial pH 5.5. At initial pH 6.0 and 4.0 biomass saccharification by *Trichoderma* SG2 cellulolytic-xylanolytic enzymes were significantly less than observed at initial pH 5.0-5.5.

Interaction with the industry for scale-up equipment

We interacted with E-fuel Corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China for procurement of equipment for scale up studies.

Manuscript preparation

Time was spent on data processing, manuscript preparation and conference presentations. One manuscript is under review for publication.

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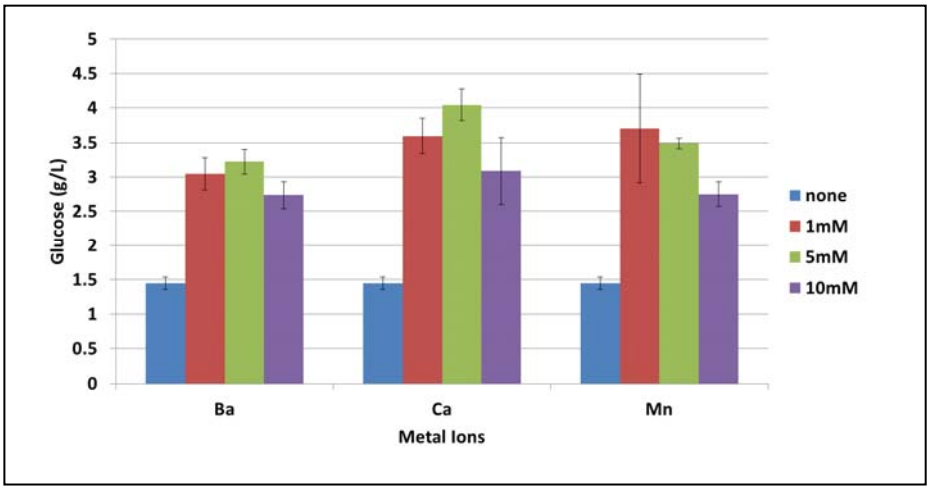

Fig. 1: Glucose produced from 2% pretreated switchgrass in 24h.

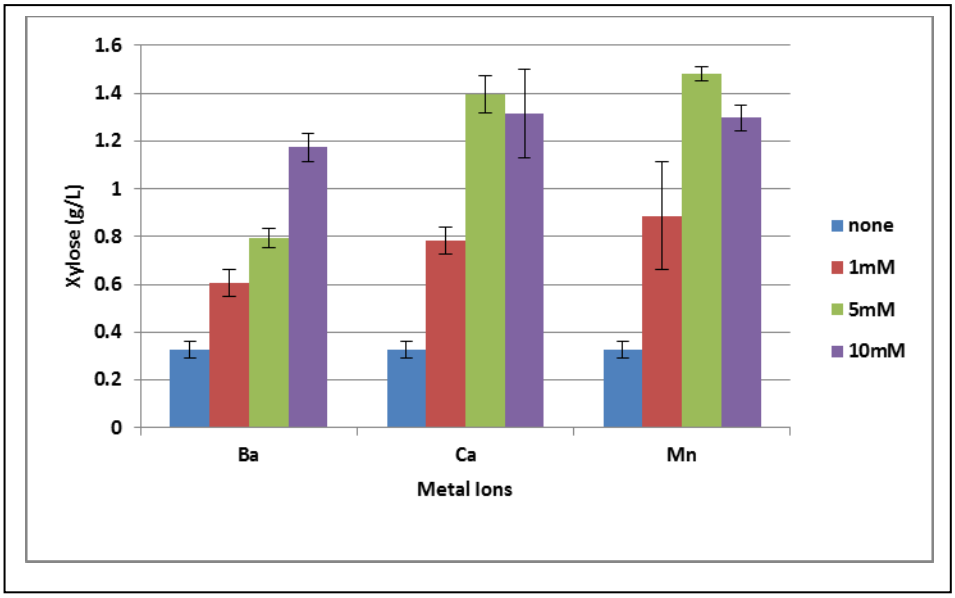

Fig. 2: Xylose produced from 2% pretreated switchgrass in 24h.

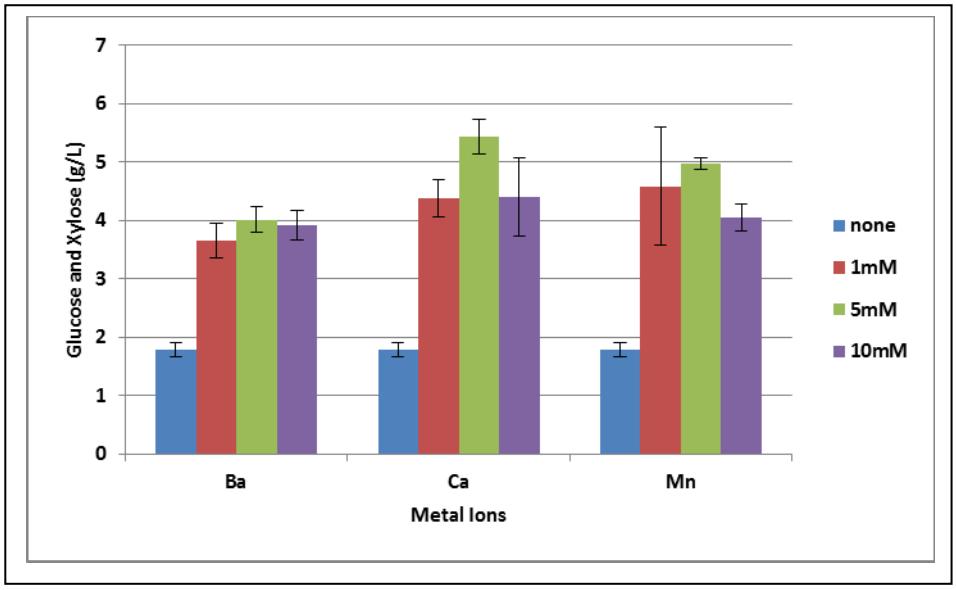

Fig. 3: Total glucose and xylose produced from 2% pretreated switchgrass in 24h.

Table 2. Relative effects of the metal ions on promotion of glucose and xylose release

Sugars	Metal Ions	Relative effects on glucose and xylose release (%)[a]		
		1mM	5mM	10mM
Glucose	Ba	210.06	222.27	188.68
	Ca	247.93	278.89	213.03
	Mn	255.24	240.75	189.72
Xylose	Ba	184.75	241.76	358.23
	Ca	238.71	425.30	401.21
	Mn	270.42	451.21	395.42
Glucose+Xylose	Ba	205.39	225.87	219.96
	Ca	246.23	305.90	247.75
	Mn	258.042	279.58	227.67

[a]Calculated relative to results (100% effect) obtained without metal ion addition

Table 3: Effect of pH on biomass saccharification by *Trichoderma* SG2 enzyme.

pH	**Reducing sugar[a] (g/L)**	**SD (g/L)**	**Relative effect (%)**
6.0	5.614	0.316	77.944
5.5	6.917	0.089	96.034
5.0	7.203	0.310	100
4.5	6.132	0.275	85.131
4.0	5.578	0.302	77.449

[a]Reducing sugar was determined by DNS method. Relative effect was calculated relative to maximum sugar yield as 100%.

Presentations

1. Rafferty, Sharla; Paulk, Andrew; Ingram, Christiane and Okeke Benedict (2014): Influence of Metal Ions on Biomass Saccharification by Cellulolytic-Xylanolytic Culture Filtrate of a New *Trichoderma* sp. SG2. American Society for Microbiology, 113th General Meeting, May 17-20, 2014, Boston, Massachusetts. Presented May 20, 2014
2. Rafferty, Sharla; Paulk, Andrew; Ingram, Christiane and Okeke Benedict (2014): Effects of Metal Ions on Cellulolytic-Xylanolytic Enzymes of a New *Trichoderma* species and Lignocellulose Biomass Conversion. Auburn Research Week 2014, April 15-17, Auburn, AL. Presented April 16, 2014.
3. Rafferty, Sharla;Paulk, Andrew; Ingram, Christiane and Okeke Benedict (2014): Metal Ion Stimulated Saccharification of Biomass by Cellulolytic-Xylanolytic Enzymes Complex of *Trichoderma* species SG2. AUM Sciences Undergraduate Research Symposium 2014, April 16, Montgomery, AL.

Conclusion

Metal ion promotion of biomass saccharification by cellulolytic and xylanolytic enzymes of *Trichoderma* species SG2 was validated by HPLC analysis of amounts of glucose and xylose produced during switchgrass biomass hydrolysis in the presence of barium, calcium and manganese. Addition of Ba, Ca and Mn to biomass saccharification reaction mixture improved glucose and xylose yield compared to the control. Overall, 5mM Ba, Ca and Mn were the most effective for glucose release. Similar results were observed with Ca and Mn for xylose release from switchgrass biomass. Improvement of glucose and xylose release by the three metal ions confirmed previous results which indicated that the metal ions promoted cellulolytic and xylanolytic enzyme activities as well as total reducing sugar release. Switchgrass biomass saccharification was pH dependent and maximum release of sugar was observed at pH 5.0-5.5.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) *Methods of soil analysis, part 2. Microbiological and biochemical properties*. Soil Science Society of America, Madison, WI, pp 407–426.
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PART 15: Research Activities of FY-14 (July 1, 2014 to September 30, 2014).
Written by: Benedict Okeke, Professor,

Introduction and Objectives

In previous report, metal ion promotion of biomass saccharification by cellulolytic and xylanolytic enzymes of *Trichoderma* species SG2 was validated by HPLC analysis of amounts of glucose and xylose produced during switchgrass biomass hydrolysis in the presence of barium, calcium and manganese. Addition of Ba, Ca and Mn to biomass saccharification reaction mixture improved glucose and xylose yield compared to the control. Overall, 5mM Ba, Ca and Mn were the most effective for glucose release. Similar results were observed with Ca and Mn for xylose release from switchgrass biomass. Improvement of glucose and xylose release by the three metal ions confirmed previous results which indicated that the metal ions promoted cellulolytic and xylanolytic enzyme activities as well as total reducing sugar release. Switchgrass biomass saccharification was pH dependent and maximum release of sugar was observed at pH 5.0-5.5.

This report presents co-production of cellulolytic-xylanolytic-amylolytic enzymes complex of *Trichoderma* species SG2 for co-saccharification of cellulosic biomass and starchy polysaccharides and optimization of temperature for switchgrass biomass saccharification by *Trichoderma* species SG2 cellulolytic-xylanolytic enzymes. Time was also spent on data processing and manuscript preparation.

Methods

Co-production of cellulolytic, xylanolytic, and amylolytic enzymes by *Trichoderma* SG2.

Trichoderma SG2 cultures were analyzed for the ability to co-produce cellulase, xylanase and amylase in liquid submerged culture. Enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of trace element solution (Focht, 1994).In independent parallel experiments, 50 mL of medium was added to a 250 mL Erlenmeyer flasks. Then, 0.25 g of pulverized switch grass (particle size ≤ 2 mm) and 0.25 g of paper powder (particle size ≤ 2 mm) were added to each flask. In parallel experiment, a combination of 0.25 g of soluble starch, 0.25 g of pulverized switch grass and 0.25 g of paper powder served as the carbon source. Flasks were plugged with foam plugs (identi-plug 46-65MM CS50) and then autoclaved at 121˚C for 20 min. After cooling to room temperature, flasks were inoculated with one potato dextrose agar (PDA) culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. The inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of *Trichoderma* sp. SG2 and incubated at 30°C for 3 days and then preserved at 4°C. Cultures were incubated at 30°C with orbital shaking (200 rpm) for 6 days. Enzyme (cell-free culture supernatant) was recovered by centrifugation (8500 rpm, 10 min, 20˚C).

Enzyme assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter

paper disc. Amylase assay mixture comprised 0.5 ml of 2% gelatinized starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Amylase assay mixture comprised 0.5 ml of 2% starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller (1959).

Co-saccharification of switch grass and starch

Switchgrass biomass was sequentially pre-treated with 1% NaOH with autoclaving at 121^{o}C for 20 min and 1% sulfuric acid with autoclaving at 121^{o}C for 20 min (FY13_Fourth_Quarter_Report). The reaction mixture comprised of 0.1g of pretreated switchgrass, 1g of 20% soluble starch solution gelatinized by autoclaving (121^{o}C for 20 min), 5.0 mL of enzyme (cell-free culture supernatant) and 50µL of 200mg/L lactrol in 15 mL centrifuge tube. The control was the same as the reaction mixture except that the crude enzyme was replaced with 5.0mL of distilled water. The reaction tubes were tightly sealed and placed horizontally on an orbital incubator and incubated for 24 hours at 50^{o}C and 80 rpm. In parallel experiment, the reaction mixture was set up without addition of starch. Control reactions were set up using water in place of the enzyme.

Effect of temperature

The effect of temperature on biomass saccharification by *Trichoderma* SG2 enzyme was examined. Saccharification reaction mixture comprised of 0.2g of pretreated switchgrass, 10.0 mL of enzyme (cell-free culture supernatant) and 100µL of 200mg/L lactrol in 125 mL Erlenmeyer flasks. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0mL of distilled water. The reactions were incubated at 50°C, 45°C, 40°C and 35°C in an orbital incubator (80 rpm) for 22 hours. Post incubation, 100 µL of digest was diluted in 900 µl of deionized water. Sugars in each sample were quantified using DNS method of Miller (1959).

Manuscript preparation

Time was spent on data processing and manuscript preparation.

Interaction with the industry for scale-up equipment

Customized equipment for scale up studies are in production by E-fuel Corporation CA, USA and Daeyoo Tech. Co. Ltd Wenzhou, China.

Results

Co-production of cellulolytic-xylanolytic-amylolytic enzymes of *Trichoderma* sp. SG2

Analysis of enzyme levels in enzyme production culture containing “switch grass and paper powder” (SP) and “switch grass, paper powder and starch” (SPS) revealed significant co-production of amylase with cellulolytic and xylanolytic enzymes (Table 1). Cellulase and xylanase production were higher in SP medium than in SPS medium. Whereas, amylase production was higher in SPS medium.

Table 1: Co-production of cellulolytic-xylanolytic-amylolytic enzymes

Enzyme	Switch grass, paper powder, and starch (SPS) medium		Switch grass and paper powder (SP) medium	
	Enzyme activity (U/mL/30min)	SD (U/mL/30min)	Enzyme activity (U/mL/30min)	SD (U/mL/30min)
Cellulase	5.16	0.49	7.31	0.57
Xylanase	28.15	3.30	32.70	1.85
Amylase	31.96	3.12	14.39	2.41

Co-saccharification of switch grass biomass and starch

The cellulolytic-xalanolytic-amylolytic enzymes complex of *Trichoderma* SG2 produced in lignocellulose (switch grass and paper powder medium) was employed for co-saccharification of switchgrass and starch (Table 2). Results show that addition of starch to lignocellulose biomass saccharification displayed an additive effect with respect to total reducing sugar yield from saccharification of combination of switchgrass and starch compared to switchgrass saccharification.

Table 2: Co-saccharification of switch grass and starch using *Trichoderma* sp. SG2 cellulolytic-xylanolytic-amylolytic cell-free culture supernatant from a lignocellulose medium without starch.

Substrates	**Reducing sugar[a] (g/L)**	**SD (g/L)**
Switch grass (20g/L) Starch (20g/L)	8.001	0.756
Switchgrass (20g/L)	6.154	0.277
Starch (20g/L)	2.543	0.208

Effect of temperature on biomass saccharification by *Trichoderma* SG2 enzyme

Figure 1 presents the effect of temperature on biomass saccharification by *Trichoderma* SG2 enzyme. Sugar yield was directly related to temperature between 35^{o}C and 50^{o}C with the maximum yield recorded at 50^{o}C. Relative effect of temperature on biomass saccharification was calculated using the maximum sugar yield observed at 50^{o}C as 100%. At 45^{o}C, 40^{o}C and 35^{o}C, relative saccharification were 90.5%, 89.3% and 83.4% respectively.

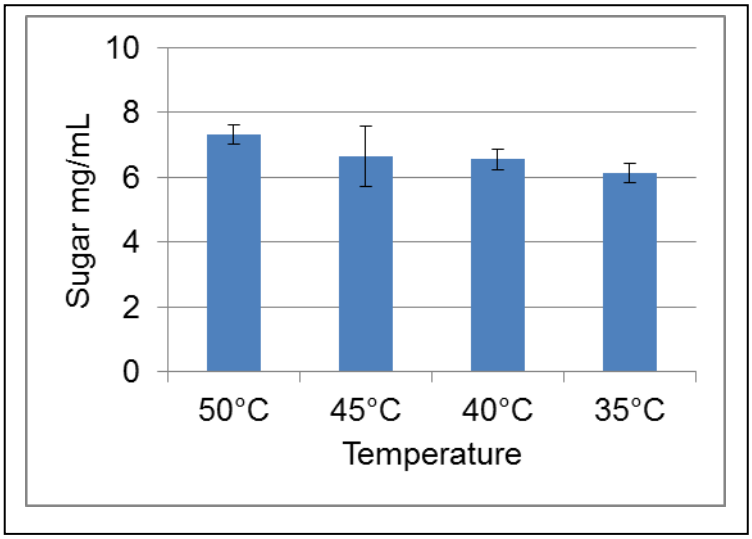

Figure 1: Effect of temperature on biomass saccharification by *Trichoderma* SG2

Publications

1. Okeke B.C (2014). Cellulolytic and xylanolytic potential of high β-blucosidase producing *Trichoderma* from decaying biomass. *Applied Biochemistry and Biotechnology*. 174:1581-98. doi: 10.1007/s12010-014-1121-x.
2. Okeke, Benedict and Nanjundaswamy, Ananda (2014). "An Efficient Process for Producing Saccharides and Ethanol from a Biomass Feedstock" (14/205,779), Cross Reference to Related Applications (61/787,039). *United States Patent Application Publication*. US 2014/0273106A1, September 18: 1-24.

Presentations

1. **Okeke, Benedict**; Rafferty, Sharla; Ingram, Christiane and Nanjundaswamy, Ananda (2014): Co-saccharification of lignocellulose biomass in conjunction with starch by cellulolytic-xylanolytic-amylolytic enzymes complex of *Trichoderma* sp. SG2. Society for Industrial Microbiology & Biotechnology Annual Meeting and Exhibition 2014 (July 19-24, 2014). Presented July 20, 2014.

Conclusions

The fungal isolate, *Trichoderma*, SG2 produces essential biomass hydrolyzing enzymes for potential application in biofuel production. Addition of starch to lignocellulosic saccharification reaction mixture displayed an additive effect in that total reducing sugar yield increased substantially compared to the saccharification of switchgrass or starch, alone. Total sugar obtained from mixed switchgrass and starch hydrolysis was close to the sum of the amounts obtained when the substrates were hydrolyzed singly. Results indicate that *Trichoderma* SG2 is a candidate organism for in-house production of cellulolytic, xylanolytic and amylolytic enzymes for saccharification of mixtures of polysaccharides in the field.

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4. Nanjundaswamy, A, Vadlani PV and Vara Prasad, PV (2011). Drought and heat stressed grain sorghum (Sorghum bicolor) does not affect the glucose and ethanol production. *Industrial Crops and Products* 33:779-782“FAQs

PART 16: Research Activities of FY-14 (October 1, 2014 to December 31, 2014).

Written by: Benedict Okeke, Professor, bokeke@aum.edu, 3342443508

Introduction and Objectives

During this quarter the construction of a custom made 250 liter engineered bioreactor system was completed by Daeyoo Tech. Co. Ltd Wenzhou, China and was delivered to our laboratory in December 2014. We explored production of cellulolytic-xylanoltyic-amylolytic enzymes by a thermotolerant fungus (CS4), isolated from a composite soil collected from four sites. In the studies, Isolate CS4 was compared to *Trichoderma* SG2 which was previously selected to be employed for enzyme production and biomass saccharification in the bioreactor.

Methods

Microorganisms

Trichoderma SG2 was previously selected for biomass saccharification in the farm deployable bioreactor under development. The thermotolerant fungus (Isolate CS4) was isolated from a composite soil collected from four sites using procedures described in FY11_Q2 and FY11_Q3 reports except that cultures were incubated at 50^{o}C to select thermophilic fungi.

Enzyme production medium and culture conditions

The enzyme production medium (EPM) comprised (g per liter): 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral element solution (Focht, 1994). Tween 80 was firstly dissolved in a boiled 50 ml aliquot of the medium and then added to the medium. Fifty milliliters of EPM was dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle size) were added. In parallel experiment 0.25 g of soluble starch was added to each flask to determine if starch addition would influence co-production of the enzymes at different incubation temperature. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of the thermotolerant fungus (CS4) and *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 6 days. Enzyme (culture supernatant) was recovered by centrifugation (8500 rpm, 20 min) using an Eppendorf centrifuge. SG2 and CS4 inocula were prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

In parallel experiment replacement of peptone with yeast extract in the EPM medium amended with 0.25% of soluble starch in addition to switchgrass and paper powder was evaluated. To this medium yeast extract (1.5g/L) was added and peptone was eliminated. The yeast extract EPM medium was also employed to evaluate the effect of increasing concentration of starch (0-0.2% and 0.2%-0.5%) on production of cellulolytic-xylanolytic-amylolytic enzymes complex of Isolate CS4 and *Trichoderma* sp. SG2. Beta glucosidase and beta-xylosidase activities of Isolate CS4 and *Trichoderma* sp. SG2 were compared using cell free culture supernatant produced using the EPM medium amended with yeast extract (1.5g/L).

Enzyme assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2003) and cooled on ice before measuring absorbance at λ_{405}. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside.

One unit of enzyme activity was 1 µmol of product released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

Saccharification of substrates

Switchgrass biomass was sequentially pretreated with 1% sodium hydroxide and 1% sulfuric acid as described in FY14_Q4 report. The pretreated switchgrass (SG) and expired wheat bread (BD) were pulverized and sieved through a 2 mm mesh sieve. A 20% (w/w) suspension of the expired bread (5g in 20 mL of water) was prepared and boiled by heating in a microwave oven at high heat for 1 min and repeated four times. The bread slurry was made up to 25 g with water. The same method was used to prepare starch (SH) solution. The reaction mixture comprised 10 ml of cell-free enzyme in 125 mL screw cap Erlenmeyer flask, 2% of each substrate, 100 µl of 200 mg/L lactrol, 100 µl of 500 mM manganese and 30 µl of 2.5% H_2SO_4 to adjust pH to approximately 5.0. Combination of the substrates (SG+BD and SG+BD+SH) were also subjected to saccharification. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0 mL of distilled water. The reactions were incubated at 50^{o}C in an orbital incubator (80 rpm) for 24 hours. Post incubation, 50 µL of digest was diluted in 950 µl of deionized water and reducing sugar was determined by the DNS method (Miller, 1959).

Manuscript preparation

Time was spent on data processing and manuscript preparation.

Interaction with the industry for scale-up equipment

Components of customized equipment for scale up studies are in production by E-fuel Corporation CA, USA.

Results

Production of cellulolytic-xylanolytic-amylolytic enzymes of the thermophilic fungus (Isolate CS4)

Production of cellulolytic-xylanolytic-amylolytic enzymes by the thermotolerant fungus (Isolate CS4) in lignocellulose medium and lignocellulose medium amended with starch was evaluated at different temperatures (Fig. 1). Optimum production of cellulase by CS4 was observed between 30-35^{o}C. Significant reduction of cellulase production was

observed at 40°C. Cellulase production was similar in lignocelluloses medium and lignocelluloses medium amended with starch. Nonetheless a slight difference in cellulase production at 40°C in lignocellulose medium and lignocellulose medium amended with starch was observed. CS4 displayed xylanase production pattern similar to cellulase production at 30-40°C in lignocellulose medium and lignocellulose medium amended with starch. Amylase production at 30-40°C in lignocellulose medium and lignocellulose medium amended with starch were similar. However, at 30°C amylase production was slightly higher in lignocellulose medium amended with starch than in lignocellulose medium without starch amendment. Peptone substitution with yeast extract displayed no significant difference in levels of cellulase, hemicellulase and amylase production (Fig. 2).

Influence of starch on production of cellulolytic-xylanolytic-amylolytic enzymes complex of isolate CS4 and *Trichoderma* SG2

The effect of different concentrations (0-0.2%) of starch on production of cellulolytic-xylanolytic-amylolytic enzymes of Isolate CS4 (Fig. 3) and *Trichoderma* sp. SG2 (Fig. 4) in lignocellulose medium was examined to determine if increasing concentration of starch would influence production of the cellulolytic-xylanolytic-amylolytic enzymes complex. Levels of cellulase and xylanase in culture supernatant of CS4 were similar at 0-0.2% starch. However, a slight decrease in xylanase activity was observed at 0.2% starch. Production of amylase by CS4 increased with addition of 0.05% starch and was stable between 0.05 to 0.2% starch in the lignocellulose medium. With *Trichoderma* SG2, cellulase production was indifferent at 0-0.2% starch whereas xylanase production decreased at 0.1-0.2% compared to 0-0.05% (Fig. 4). Production of amylase by *Trichoderma* SG2 in the lignocellulose medium increased with increasing concentration of starch reaching maximum at 0.2% (Fig 4).

Further studies examined the effect of higher concentrations (0.2-0.5%) of starch on production of cellulolytic-xylanolytic-amylolytic enzymes of Isolate CS4 (Fig. 5) and *Trichoderma* sp. SG2 (Fig. 6) in lignocellulose medium. Generally, increasing the concentration of starch (0.2-0.5%) did not increase enzyme production in lignocellulose medium. Xylanase production by SG2 was consistently less at 0.2% starch in two studies at 0-0.2% and 0.2-0.5%. Interestingly, at 0.2% starch amylase production by *Trichoderma* SG2 substantially increased suggesting an inverse relationship between xylanase and amylase production at the onset of increased induction of amylase production by SG2. With CS4 a similar effect, though not strong, was observed upon addition of 0.3-0.5% starch to lignocellulose medium. This observation, however, needs further studies.

CS4 is thermotolerant and strongly produces cellulase, xylanase and amylase. However, a comparative assay revealed that *Trichoderma* SG2 produced much more β-glucosidase and β-xylosidase than Isolate CS4 (Fig. 7).

Co-saccharification of switchgrass biomass and expired bread

We had shown in previous reports that the new *Trichoderma* sp SG2 simulatneously produces cellulolytic, xylanolytic and amylolytic enzymes and also demonstrated that addition of starch to switchgrass biomass displayed an additive effect in that the sugar yield was similar to the sum of sugar yield from separate saccharification of switchgrass and starch by cell free enzyme of *Trichoderma* SG2. Pure starch is expensive, thus we explored use of expired bread as a carbohydrate waste to augment sugar yield in biomass saccharification by *Trichoderma* SG2 and Isolate CS4 cellulolytic-xylanolytic-amylolytic enzymes. Both strains displayed similar yield of sugar from the substrates and their combinations (Fig. 8). Interestingly, total sugar yield from combinations of the substrates was similar to the sum of sugar yield from separate saccharification of each substrate. When 2% starch was added to a combination of switch grass and expired bread, a similar additive effect in sugar yield was

observed. Considering an initial concentration of approximately 20g/L (2%) for each substrate and that actual cellulose, hemicelluloses and starch content of the substrates are less than 20g/L, more than 50% conversion was observed in the 24 h saccharification.

Farm deployable bioreactor system

The 250 liter engineered bioreactor was received from Daeyoo Tech. Co. Ltd Wenzhou, China, in December, 2014. Engineering drawings of the farm deployable bioreactor system (Fig. 9) and sand filter (Fig. 10) developed for low cost separation of biomass from fermentation broth produced in the farm deployable bioreactor system are presented. Installation of the 250 liter bioreactor system is in progress.

Publications

Benedict C. Okeke*, Rosine W. Hall, Ananda Nanjundaswammy, M. Sue Thomson, Yasaman Deravi, Leah Sawyer, Andrew Prescott. Study on selection and molecular characterization of cellulolytic-xylanolytic fungi from surface soil-biomass mixtures from Black Belt sites. *Microbiological Research.* In revision.

Conclusions

The construction of a customized farm deployable bioreactor system was completed and delivered by Daeyoo Tech. Co. Ltd Wenzhou, China. The thermotolerant fungus (Isolate CS4) is a strong producer of cellulolytic-xylanolytic-amylolytic enzymes. Levels of cellulase and xylanase activities in culture supernatant of CS4 are similar to the levels observed in cultures of the previously selected strain, *Trichoderma* sp. SG2. However, CS4 produces more amylase, whereas SG 2 produces much more β-glucosidase and β-xylosidase. Thus *Trichoderma* sp. SG2 still stands as the candidate organism for enzyme production and biomass saccharification in the farm deployable bioreactor system.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, WI, pp 407–426.
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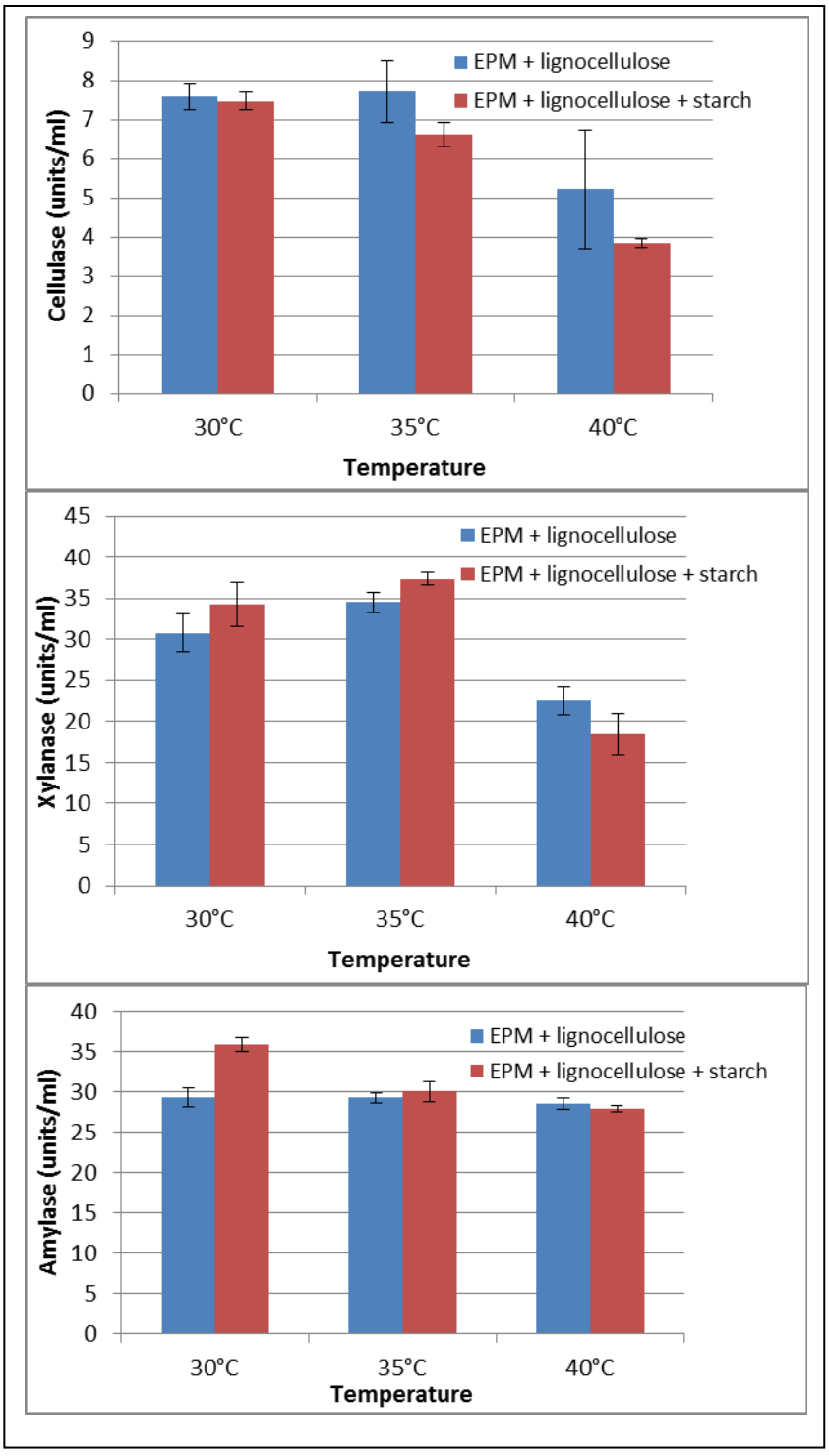

Fig. 1: Production of cellulolytic-xylanolytic-amylolytic enzymes complex of a thermotolerant mold (Isolate CS4). EPM is the enzyme production medium described in methods.

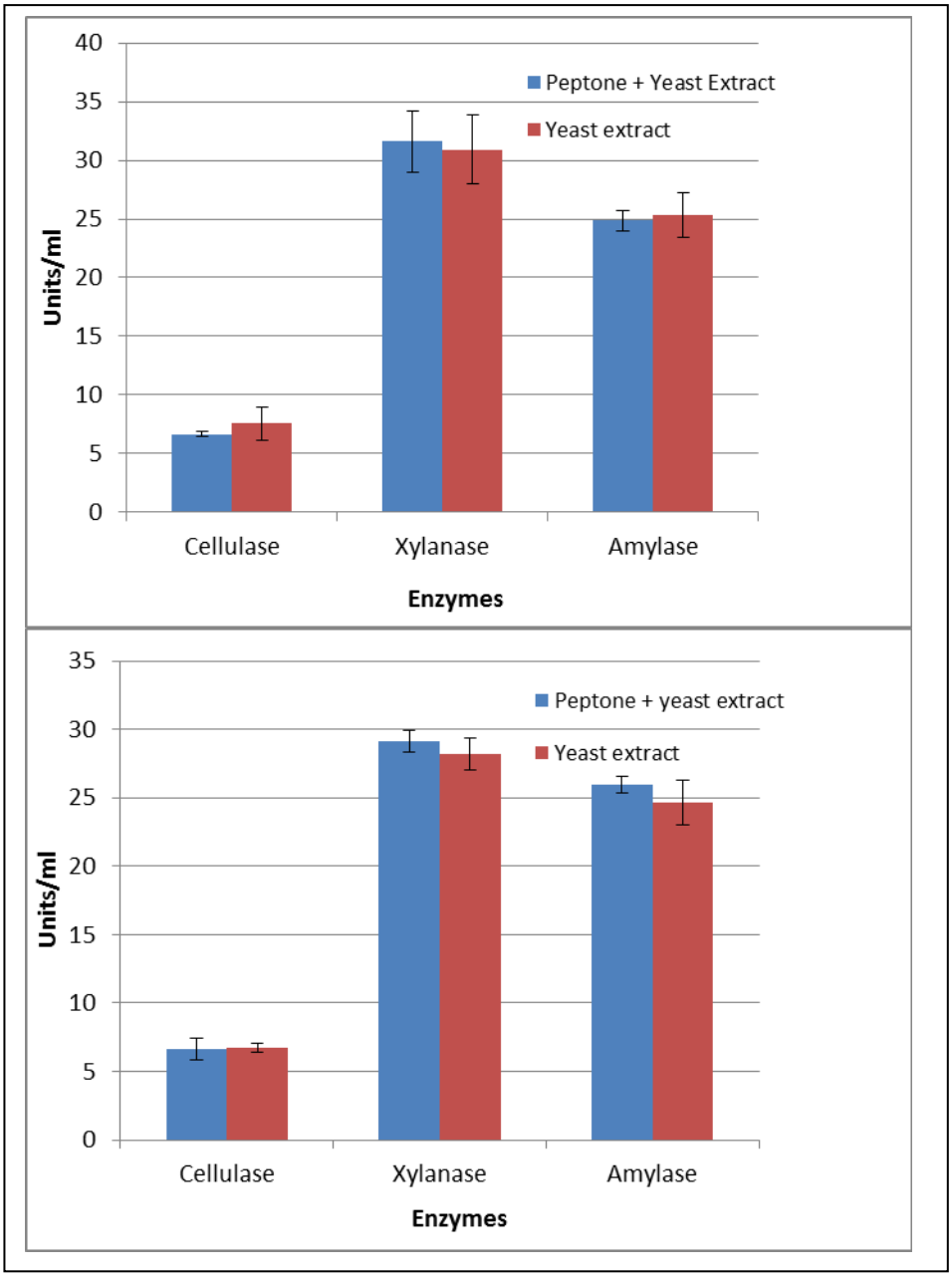

Fig. 2: Effect of peptone replacement with yeast extract on enzyme production. The enzyme production medium (EPM) contained either peptone (1g/L) and yeast extract (0.5g/L) or yeast extract (1.5g/L) alone.

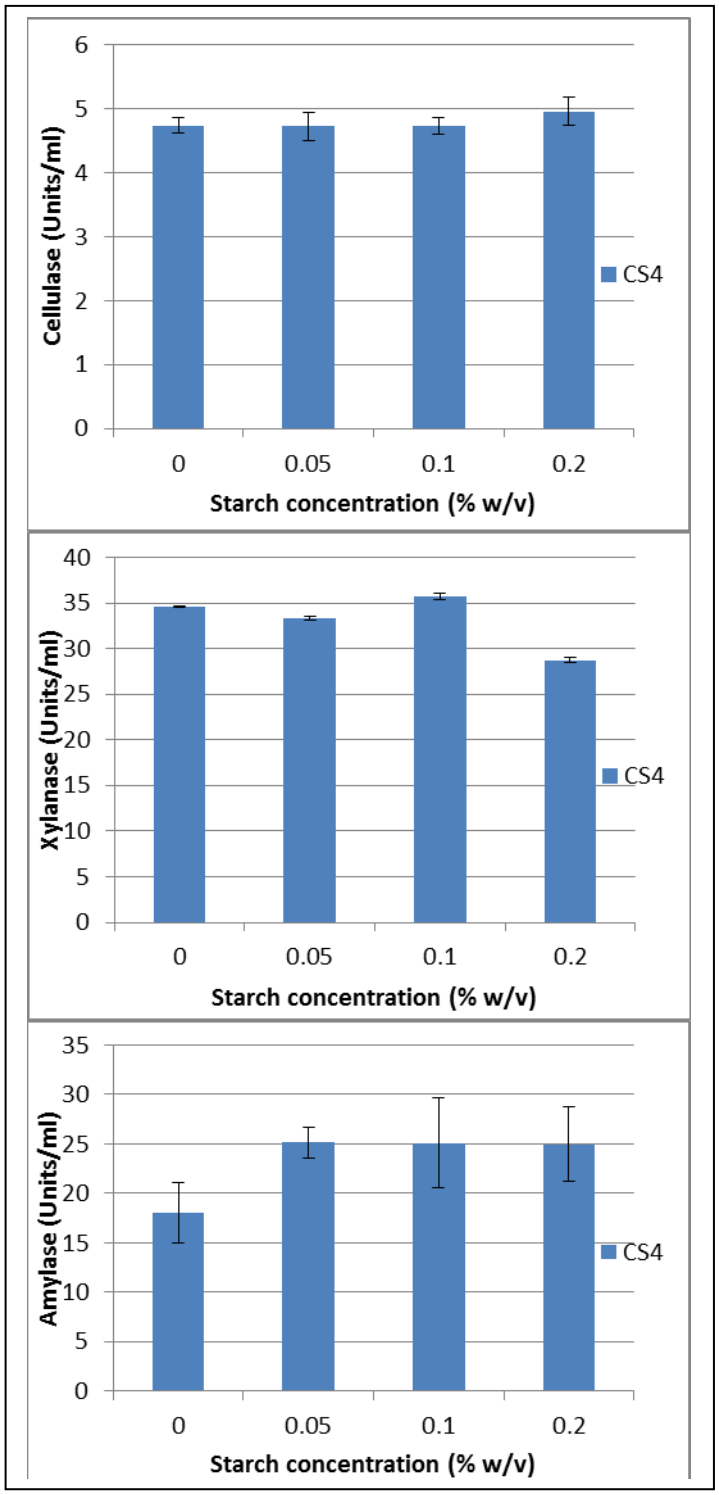

Fig 3. Effect of starch concentration (0-0.2%) on induction of cellulolytic-xylanolytic-amylolytic enzymes complex of Isolate CS4.

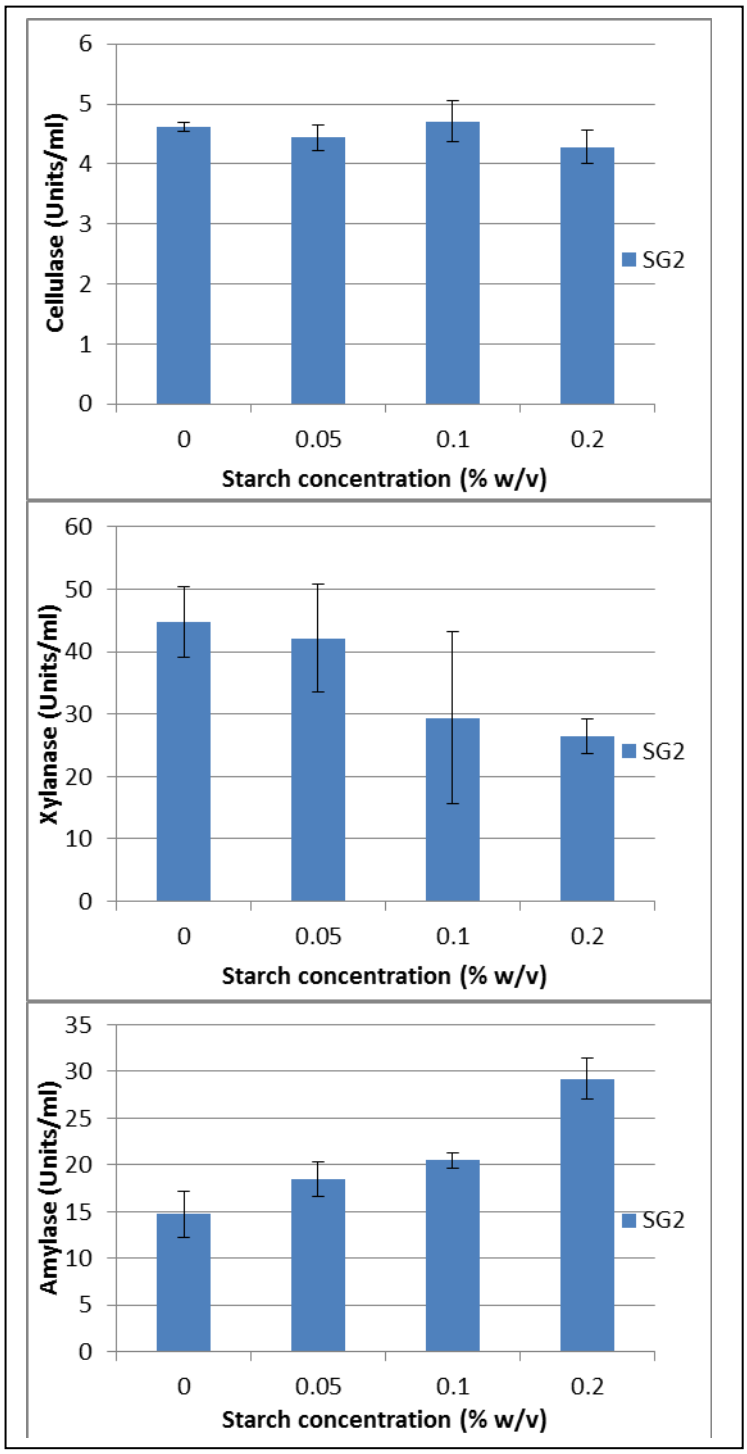

Fig 4. Effect of starch concentration (0-0.2%) on induction of cellulolytic-xylanolytic-amylolytic enzymes complex of *Trichoderma* SG2

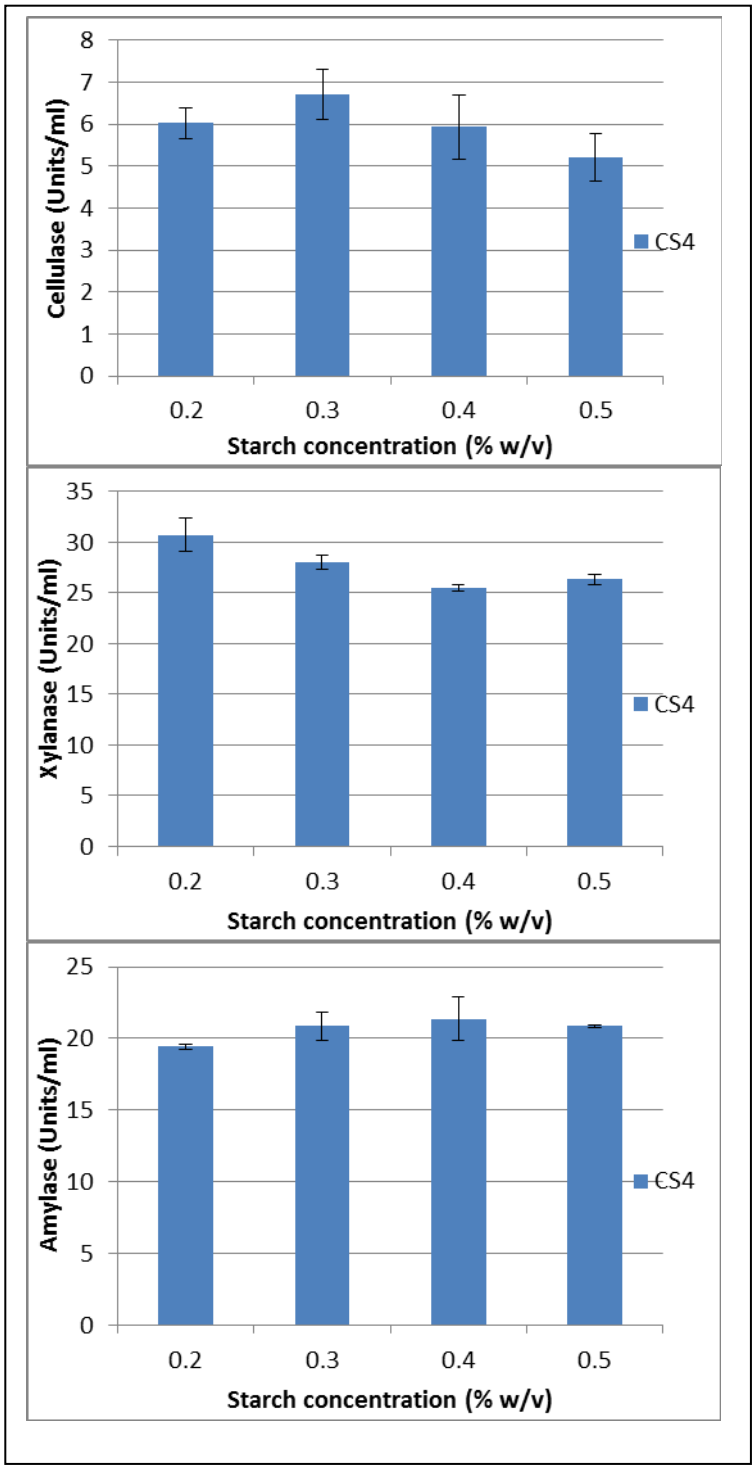

Fig 5. Effect of starch concentration (0.2-0.5%) on induction of cellulolytic-xylanolytic-amylolytic enzymes complex of Isolate CS4.

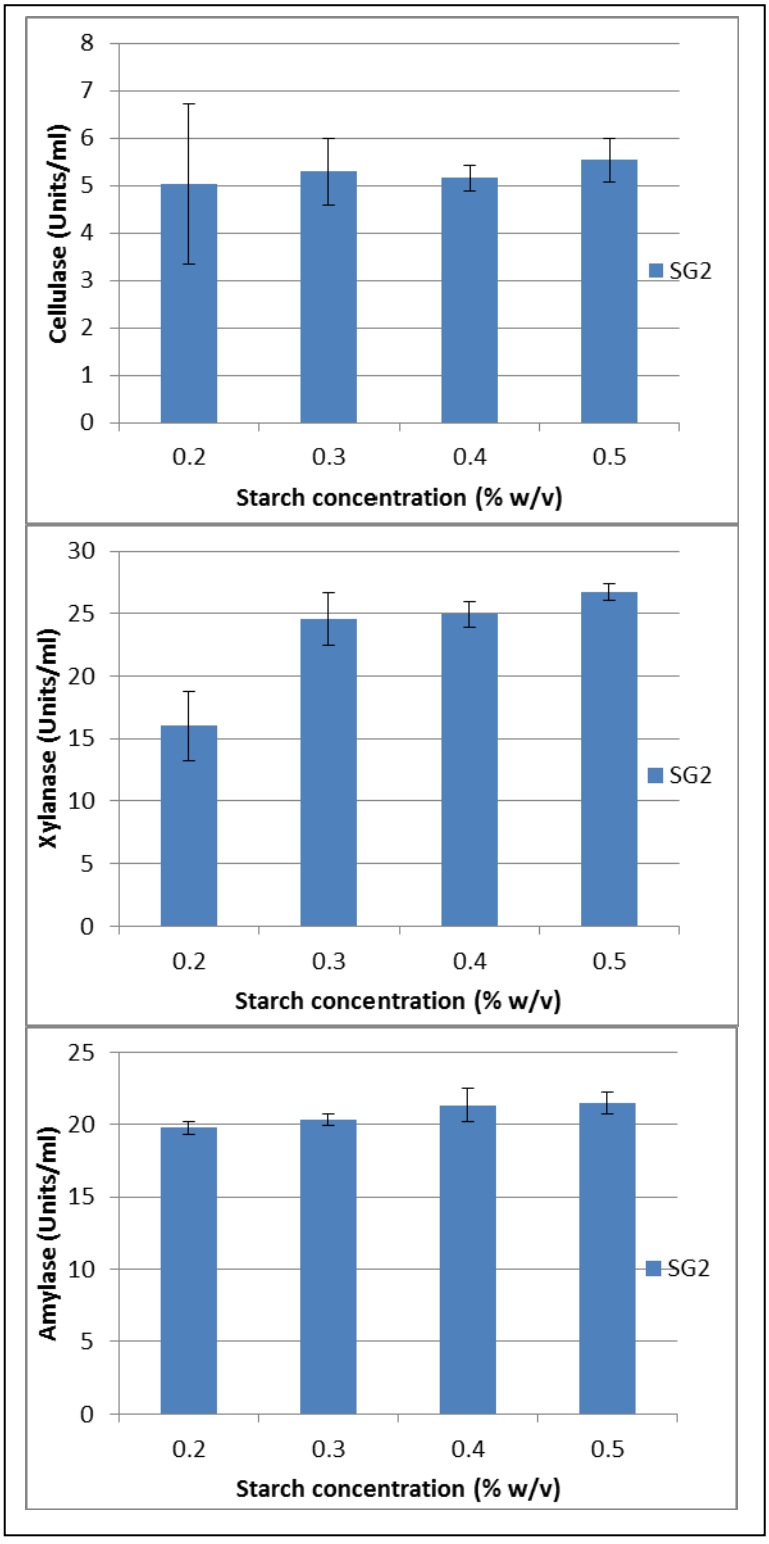

Fig 6. Effect of starch concentration (0.2-0.5%) on induction of cellulolytic-xylanolytic-amylolytic enzymes complex of *Trichoderma* SG2

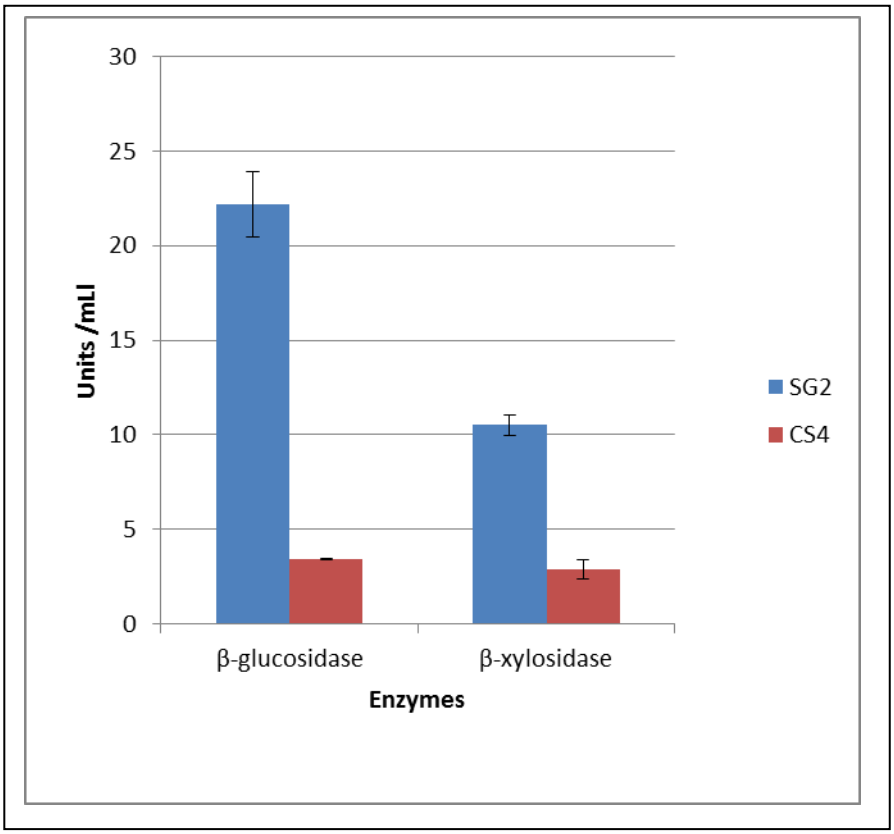

Fig. 7: Comparison of β-glucosidase and β-xylosidase of SG2 and CS4.

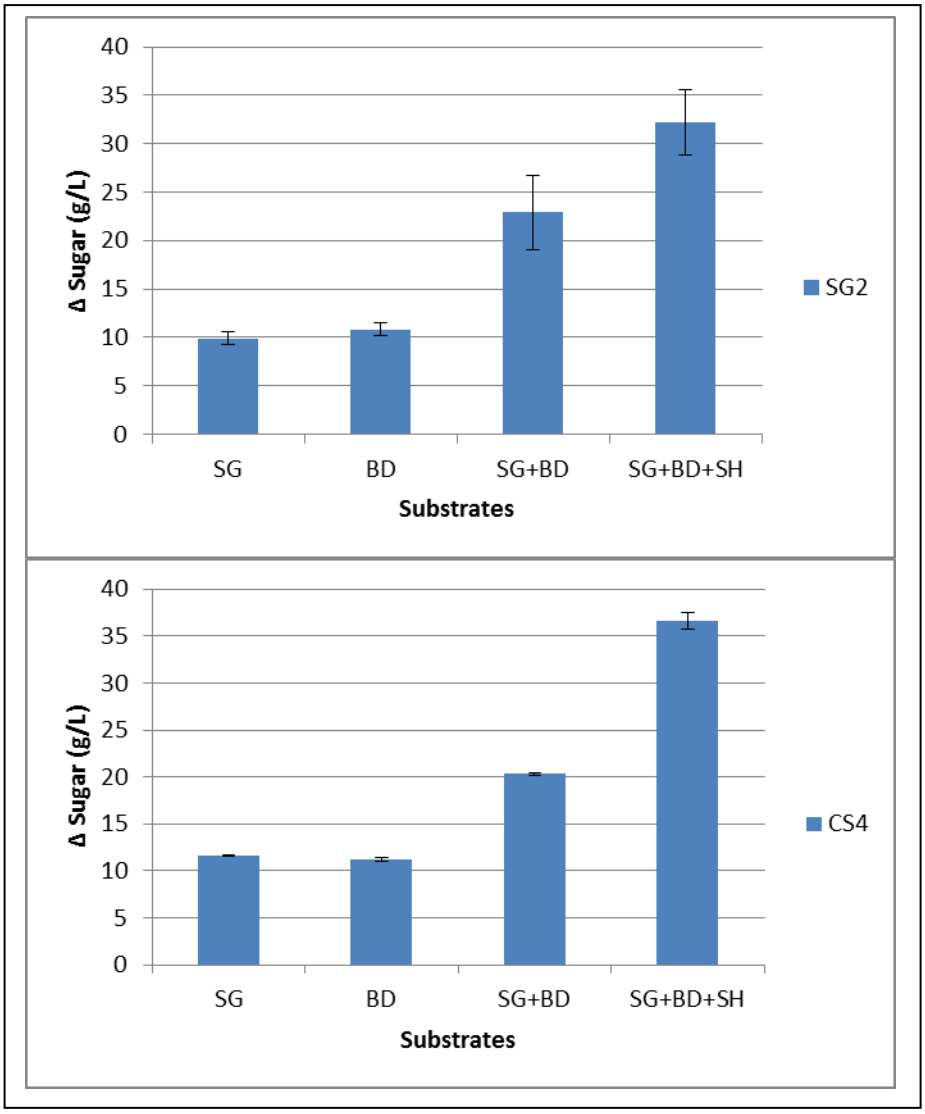

Fig 8. Co-saccharification of switchgrass, expired bread and their combinations. SG: switchgrass (2% in 10 ml enzyme), BD: expired bread (2% in 10 ml enzyme), BD+SG (2% of each in 10 ml enzyme), SH: starch, SG+BD+SH (2% of each in 10 ml enzyme). Δ sugar: the difference between treatments and substrate control. DNS method does not detect sucrose.

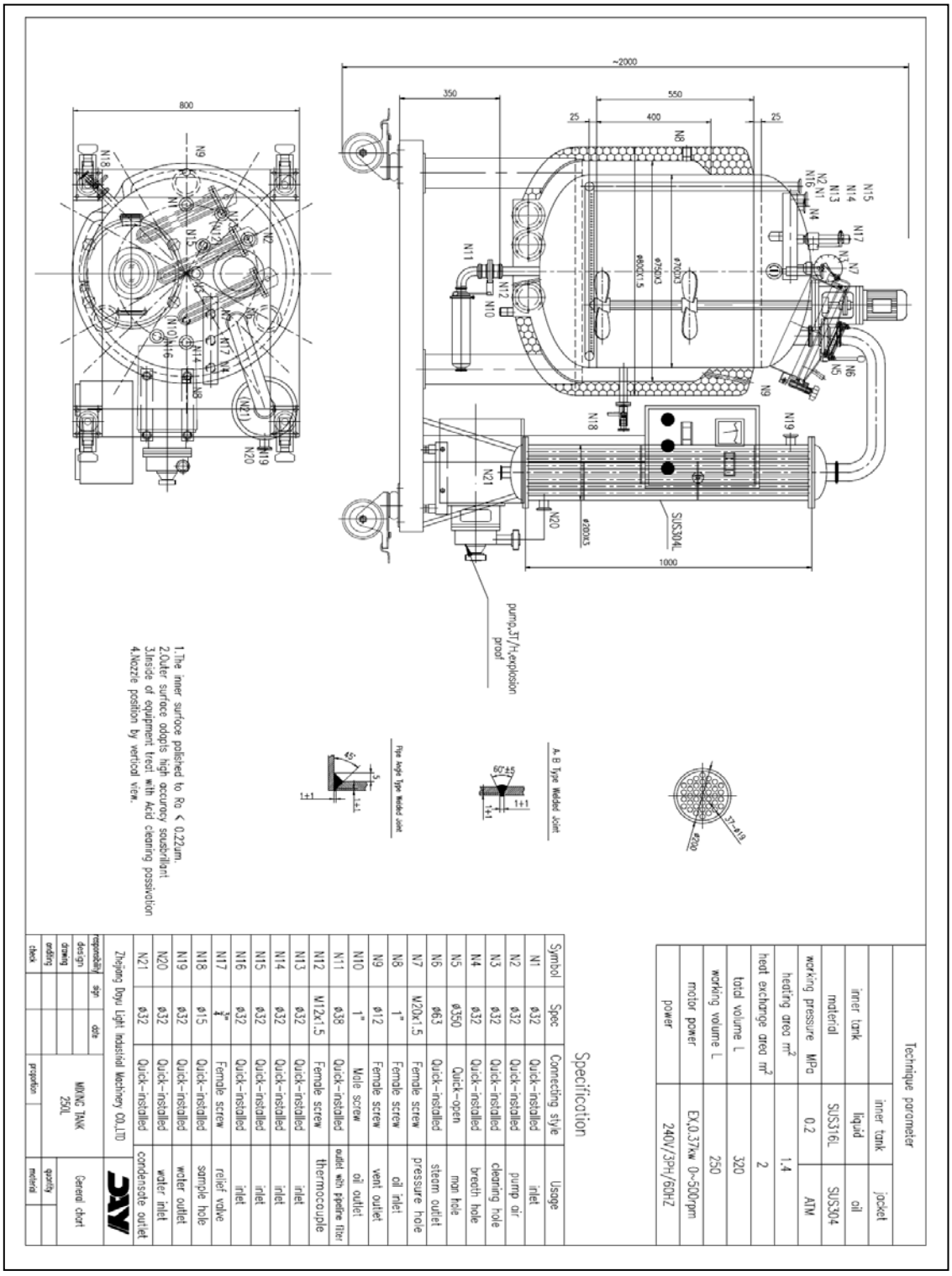

Fig. 9: Engineering drawing of the farm deployable bioreactor system.

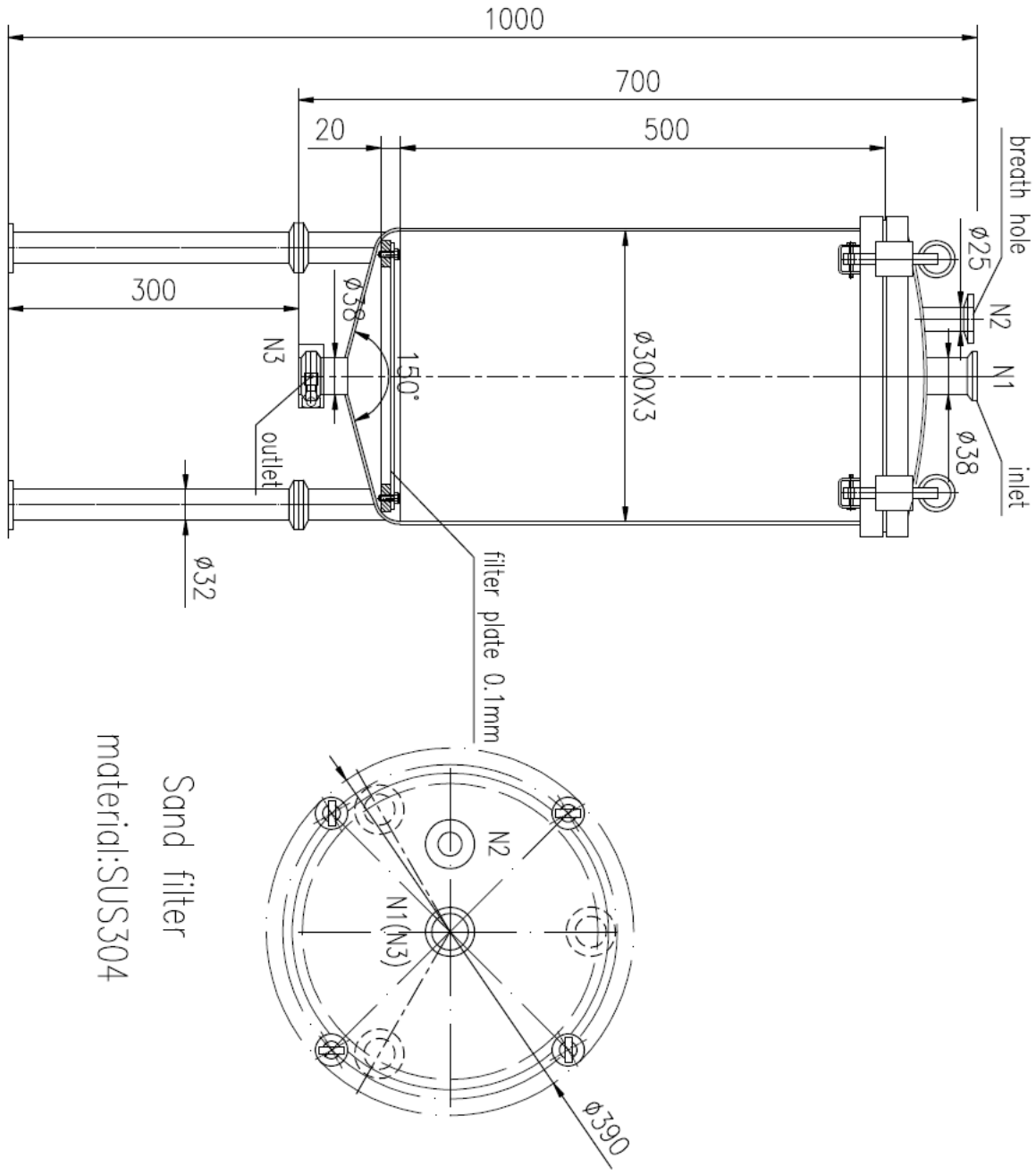

Fig. 10: Engineering drawing of a sand filter developed for biomass separation from the fermentation broth of the farm deployable bioreactor system.

PART 17: Research Activities of FY-15 (January 1, 2015 to March 31, 2015).
Written by: Benedict Okeke

Introduction and Objectives

In the previous quarter, a custom made 250 liter engineered bioreactor system was completed and delivered by Daeyoo Tech. Co. Ltd Wenzhou, China. In this quarter, the distillation component for further processing of ethanol from the bioreactor system was completed and delivered by E-fuel Corporation CA, USA. Following receipt of all components; set-up of the model farm deployable bioreactor system and measures for safety compliance are in progress. In scale up of fermentation, the cost of nutrients is a critical factor. In the previous quarter we demonstrated that peptone can be replaced with yeast extract in the previously optimized enzyme production medium; and comparable enzyme yield was achieved. Proflo is an inexpensive organic nitrogen source compared to yeast extract. We explored the influence of different concentrations of proflo on enzyme production in comparison with yeast extract. The effects of combinations of proflo, yeast extract and peptone on enzyme production were also examined.

Methods

Influence of different concentrations of proflo and yeast extract on production of enzymes.

The basal medium (BM) comprised (g per liter) of 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral elements solution (Focht, 1994). Tween 80 was firstly dissolved in an aliquot of the basal medium placed in a 50 ^oC water bath; and then added to the medium. Fifty milliliters of the basal medium containing 0.5-1.5g/L proflo and 0.5-1.5g/L yeast extract were in parallel experiments dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug (identi-plug 46-65MM CS50, VWR Cat # 60882-205 3) and then autoclaved at 121^oC for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2 or the thermotolerant fungus (CS4), obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^oC with orbital shaking (200 rpm) for 6 days. Enzyme (culture supernatant) was recovered by centrifugation (7500 rpm, 20 min) using an Eppendorf centrifuge. SG2 and CS4 inocula were prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30^oC for 3 days and then preserved at 4^oC. In another experiment with SG2, the effects of high concentration of proflo and yeast extract (1.5-5%) on enzymes production were examined.

Influence of combination of proflo, yeast extract and peptone on production of enzymes

Fifty milliliters of the basal medium (BM) containing 0.5g/L yeast extract and 1.0g/L peptone (**YEP**), 0.5g/L yeast extract and 1.5g/L proflo (**YPR**), 0.5g/L peptone and 1.5g/L proflo (**PPR**), or 1.5g/L proflo (**PRO**) or 0.5g/L yeast extract, 1.0g/L peptone and lactrol (**YPL**) or the basal medium without organic nitrogen (**NON**), were dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug and then autoclaved at 121^oC for 20 min. Culture conditions and enzyme recovery were as described above.

Enzyme assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2003) and cooled on ice before measuring absorbance at λ_{405}. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside.

One unit of enzyme activity was 1 µmol of product released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

Interaction with the industry for scale-up equipment

Time was spent interacting with E-fuel Corporation CA, USA, on completion of construction of the distillation component of the bioreactor system.

Installation of the bioreactor

We obtained quotes for installation requirements including electric power, spillage containment system, waste water drainage to the sewer and fire warning system. Power panel, 220-240 volts was installed and power was connected to the three heaters, the rotor and three pumps of the bioreactors system. A spillage containment concrete curb was built around the biroreactor system.

Results

Influence of different concentrations of proflo and yeast extract on enzymes production

The influence of low concentrations (0.5-1.5 g/L) of proflo and yeast extract on cellulase production by SG2 and CS4 are summarized in Fig. 1A-D. In general, cellulase production varied only slightly at these concentrations. Nonetheless, the highest activity of cellulase in cultures of SG2 and CS4 occurred at 1.5 g/L proflo (Fig 1A) and g/L proflo (Fig. 1B); respectively. Optimum production of cellulase by SG2 and CS4 were observed at 1.5 g/L yeast extract (Fig 1C and D). Xylanase production by SG2 varied slightly in media amended with 0.5-1.5 g/L proflo (Fig. 2A), with optimum production at 0.5 g/L proflo. CS4 displayed higher xylanase production at 1.5 g/L proflo (Fig. 2B). Xylanase production by SG2 and CS4 varied only slightly in media amended with 0.5-1.5 g/L yeast extract (Fig. 2C and D). However, 0.5 g/L yeast extract promoted more xylanase production by SG2 (Fig. 2C). The highest xylanase activity of CS4 occurred in media amended with 1 g/L yeast extract (Fig. 2D). Amylase production by SG2 and CS4 varied only slightly in media amended with 0.5-1.5 g/L proflo and yeast extract (Fig 3A-D). SG2 produced slightly more amylase at 0.5 g/L proflo (Fig. 3A); whereas CS4 produced more amylase at 1.5 g/L proflo (Fig. 3B). Amylase production SG2 and CS4 were highest in medium amended with 1 g/L yeast extract (Fig. 3C).and 0.5 g/L yeast extract (Fig. 3D), respectively.

β-glucosidase and activities of SG2 and CS4 generally increased with increasing concentration (0.5 to 1.5 g/L) of yeast extract and proflo (Fig 4A-D). β-xylosidase production by SG2 was only slightly different at 0.5 to 1.5 g/L proflo (Fig 5A); whereas β-xylosidase

production by CS4 increased with increasing concentration of proflo (Fig 5B). β-xylosidase production by SG2 were only slightly different at 0.5 to 1.5 g/L yeast extract (Fig 5C), with optimum production at 1 g/L. β-xylosidase production in medium amended with yeast extract was generally higher at 0.5 g/L. SG2 produced much more β-glucosidase and β-xylosidase than CS4 under all conditions examined.

The influence of high concentrations (1.5 to 5 g/L) of proflo and yeast extract on enzyme production by SG2 was examined (Fig. 6 to 10). Cellulase production was highest at 1.5 to 2% proflo and decreased with increasing concentration of proflo (Fig. 6A). Xylanase production at 2 to 5 g/L proflo were similar and optimum production occurred at 1.5 g/L proflo (Fig. 6B). Production of amylase was highest at 1.5 to 2 g/L proflo and decreased with increasing concentration of proflo (Fig. 6C). Similar levels of cellulase were observed in cultures at 1.5 to 3 g/L yeast extract with maximum activity at 3 g/L (Fig. 7A). Cellulase activity decreased substantially at 4 to 5 g/L yeast extract. Production of xylanase were similar at 1.5 to 3 g/L yeast extract and decreased substantially at 4 to 5 g/L yeast extract (Fig. 7B). Amylase production was inversely related to the concentration of yeast extract (Fig. 7C). Maximum production of β-glucosidase occurred at 3g/L proflo (Fig 8A) and yeast extract (Fig 8B). β-xylosidase production was highest at 1.5 g/L proflo (Fig 9A) and 2 g/L yeast extract (Fig 9B).

Influence of combination of proflo, yeast extract and peptone on enzymes production

The influence of mixtures of proflo, yeast extract and peptone on production of cellulolytic-xylanolytic enzymes by SG2 was examined. A combination of 0.5 g/L yeast extract and 1.5 g/L proflo (medium YPR) proved most effective for cellulase production (Fig 10A). Cellullase production was substantially less in medium without organic nitrogen supplementation (NON). Similar levels of xylanase production occurred in YEP, YPR, PPR and YPL media (Fig 10B). Significant production of xylanase was observed in the medium which was not amended with organic nitrogen sources (NON). Amylase production was generally similar in the six media examined (Fig 10C). β-glucosidase activity was highest in YPR medium followed by PPR medium; whereas similar levels of β-glucosidase occurred at in PRO, YPL and YEP media (Fig 11A). β-xylosidase activities in the six media were generally similar (Fig 11B).

Publications

Benedict C. Okeke*, Rosine W. Hall, Ananda Nanjundaswammy, M. Sue Thomson, Yasaman Deravi, Leah Sawyer, Andrew Prescott. Study on selection and molecular characterization of cellulolytic-xylanolytic fungi from surface soil-biomass mixtures from Black Belt sites. *Microbiological Research.* In Press.

Conclusions

The construction of all components of the customized farm deployable bioreactor system was completed and delivered. Comparative evaluation of organic nitrogen sources proflo, yeast extract and peptone revealed that a combination of 0.5 g/L yeast extract and 1.5 g/L proflo (YPR) was generally more effective for production of cellulase and β-glucosidase production. However, the results are comparable to the previously optimized enzyme production medium in which yeast extract and peptone (YEP) were the organic nitrogen sources. The advantage of YPR is that proflo is a cheap organic nitrogen source; which is a major economic advantage in scale-up fermentation.

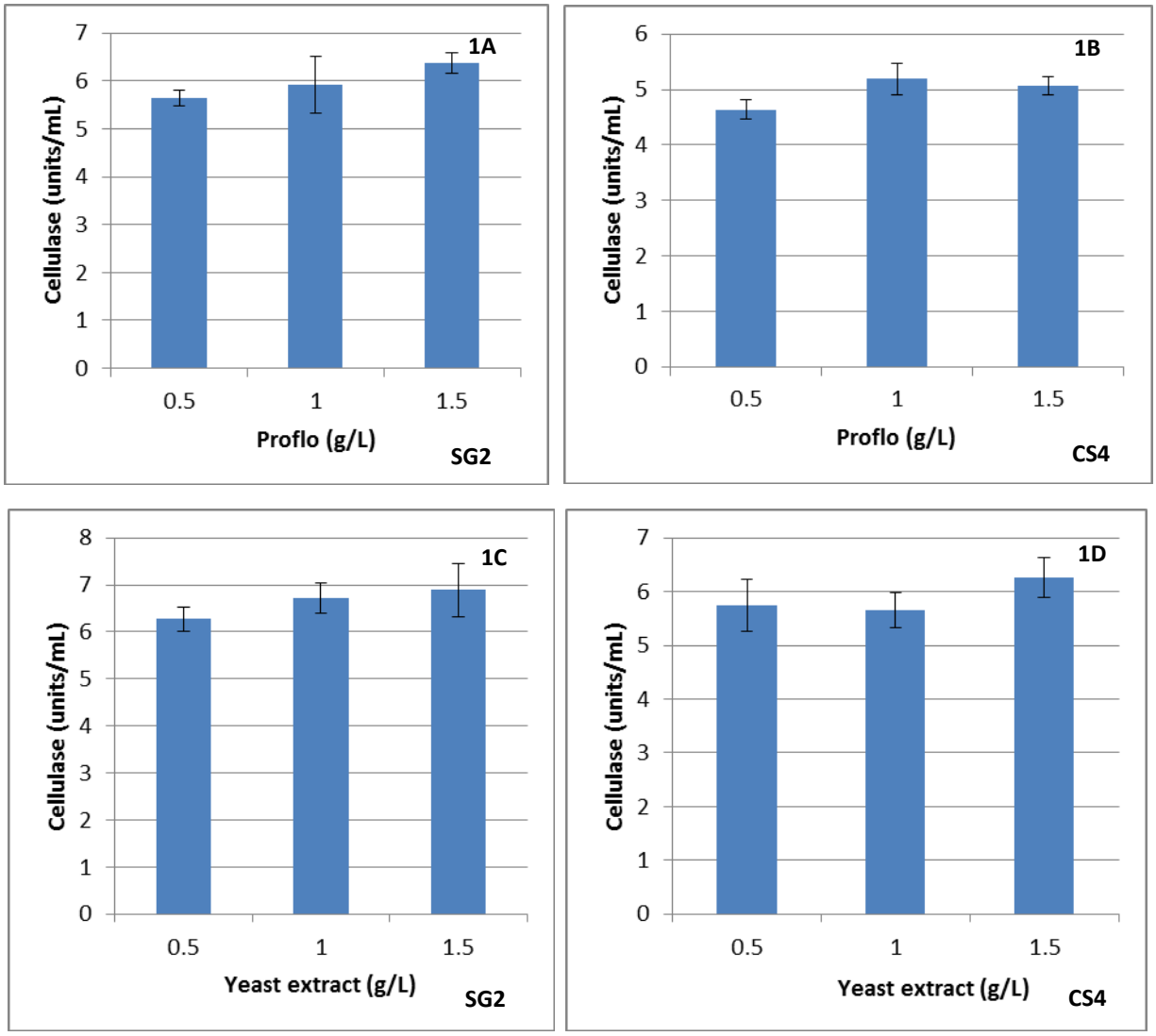

Fig 1A-D: Influence of low concentrations of proflo and yeast extract on production of cellulase of SG2 and CS4.

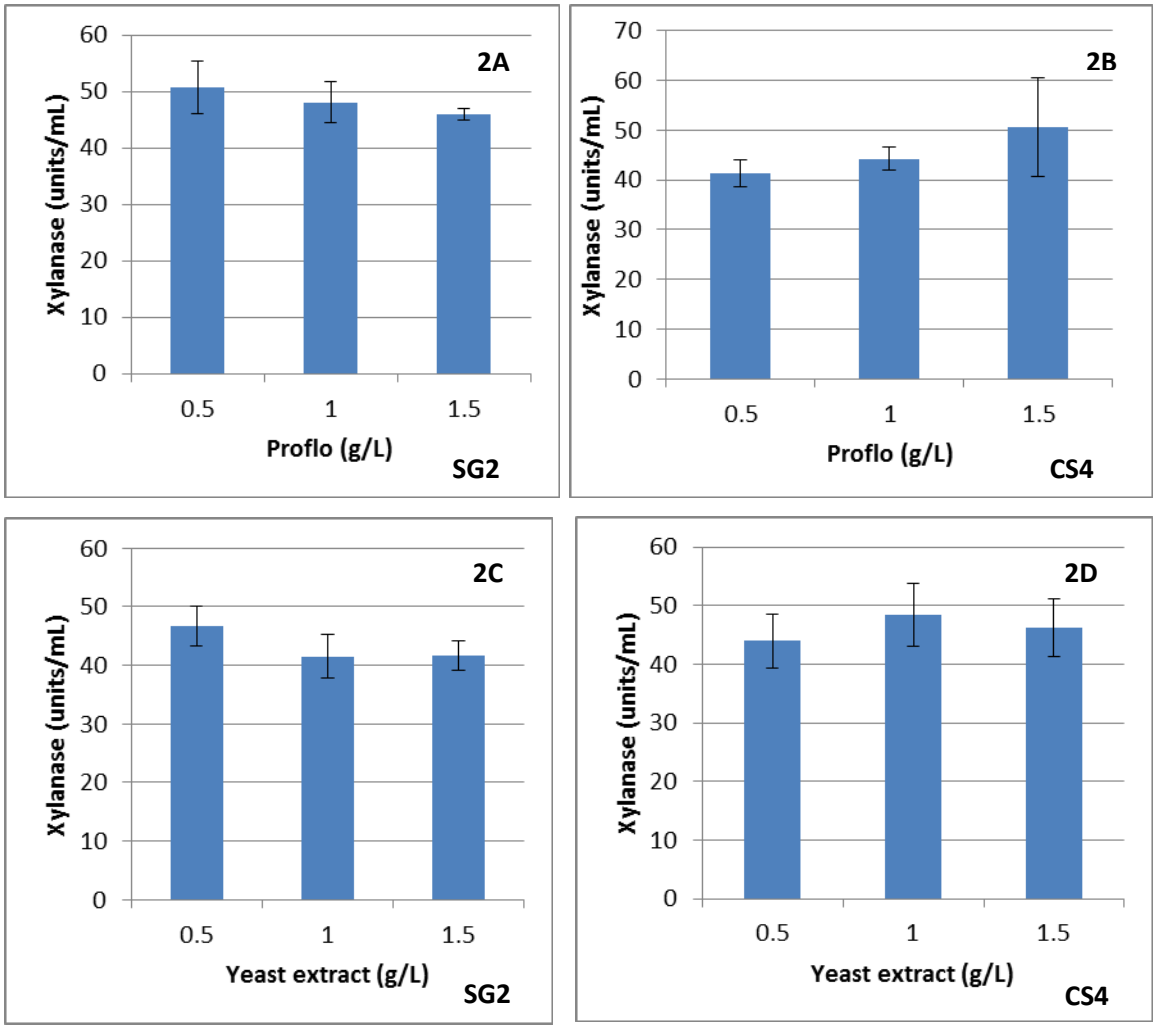

Fig 2A-D: Influence of low concentrations of proflo and yeast extract on production of xylanase of SG2 and CS4.

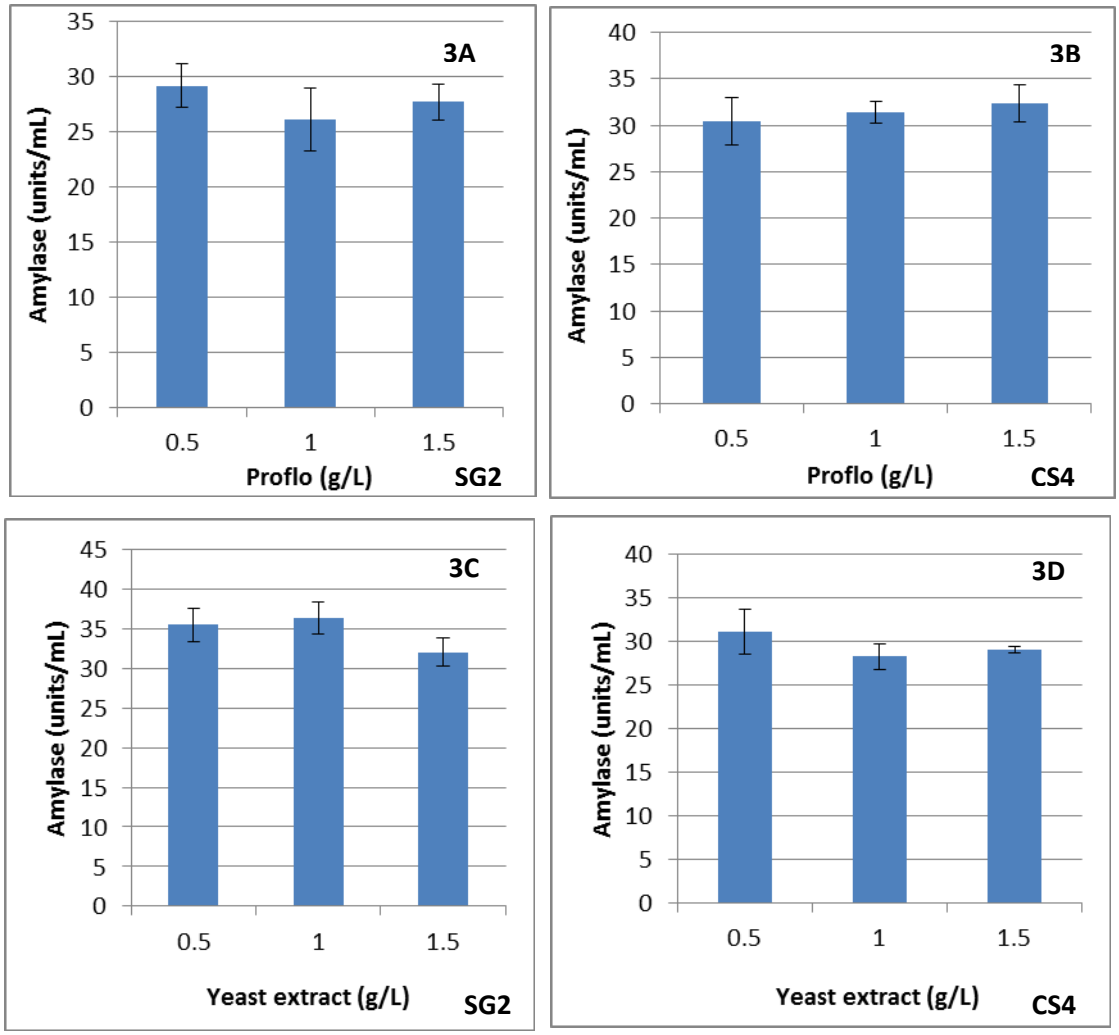

Fig 3A-D: Influence of low concentrations of proflo and yeast extract on production of amylase of SG2 and CS4.

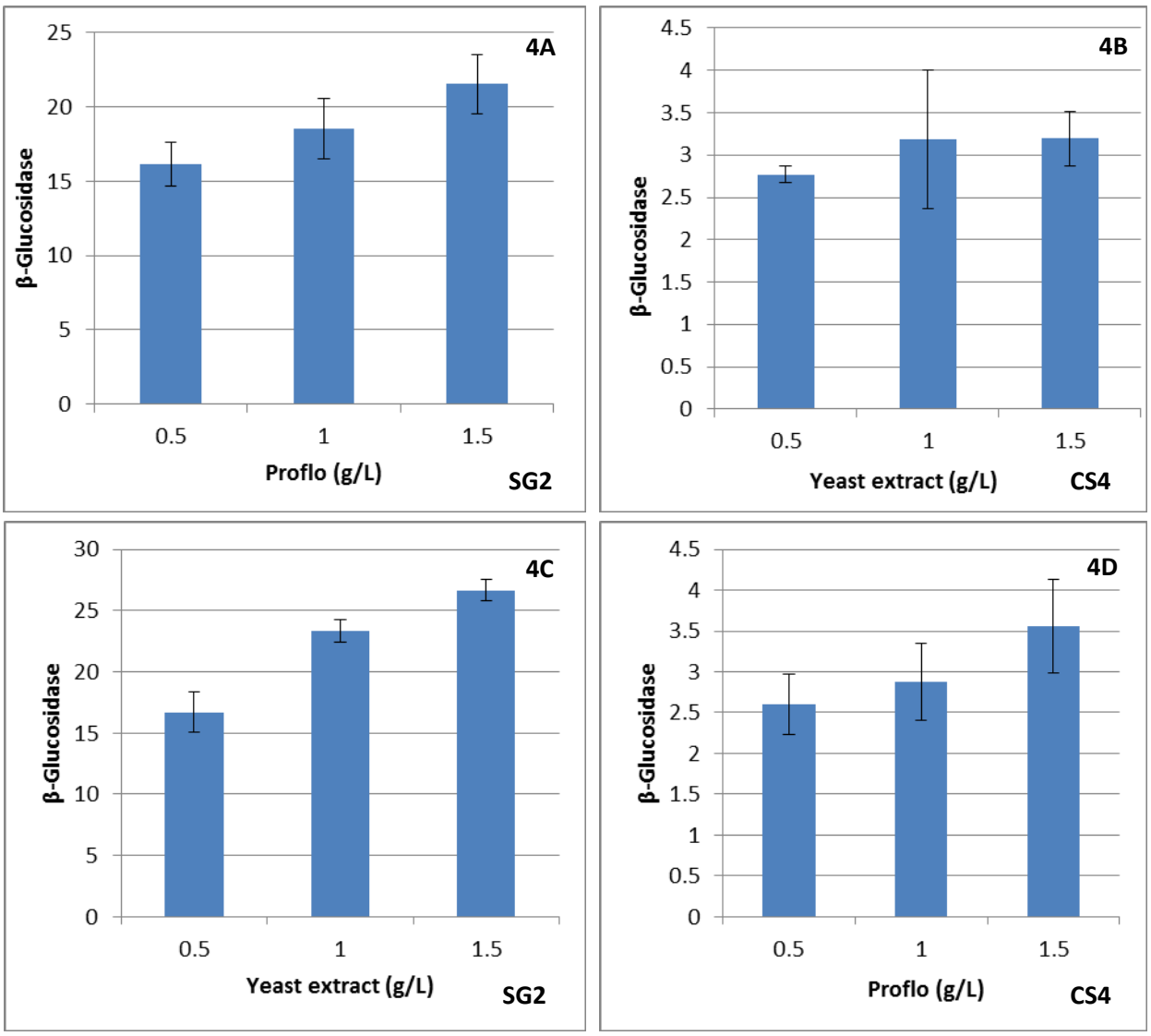

Fig 4A-D: Influence of low concentrations of proflo and yeast extract on production of β-glucosidase of SG2 and CS4.

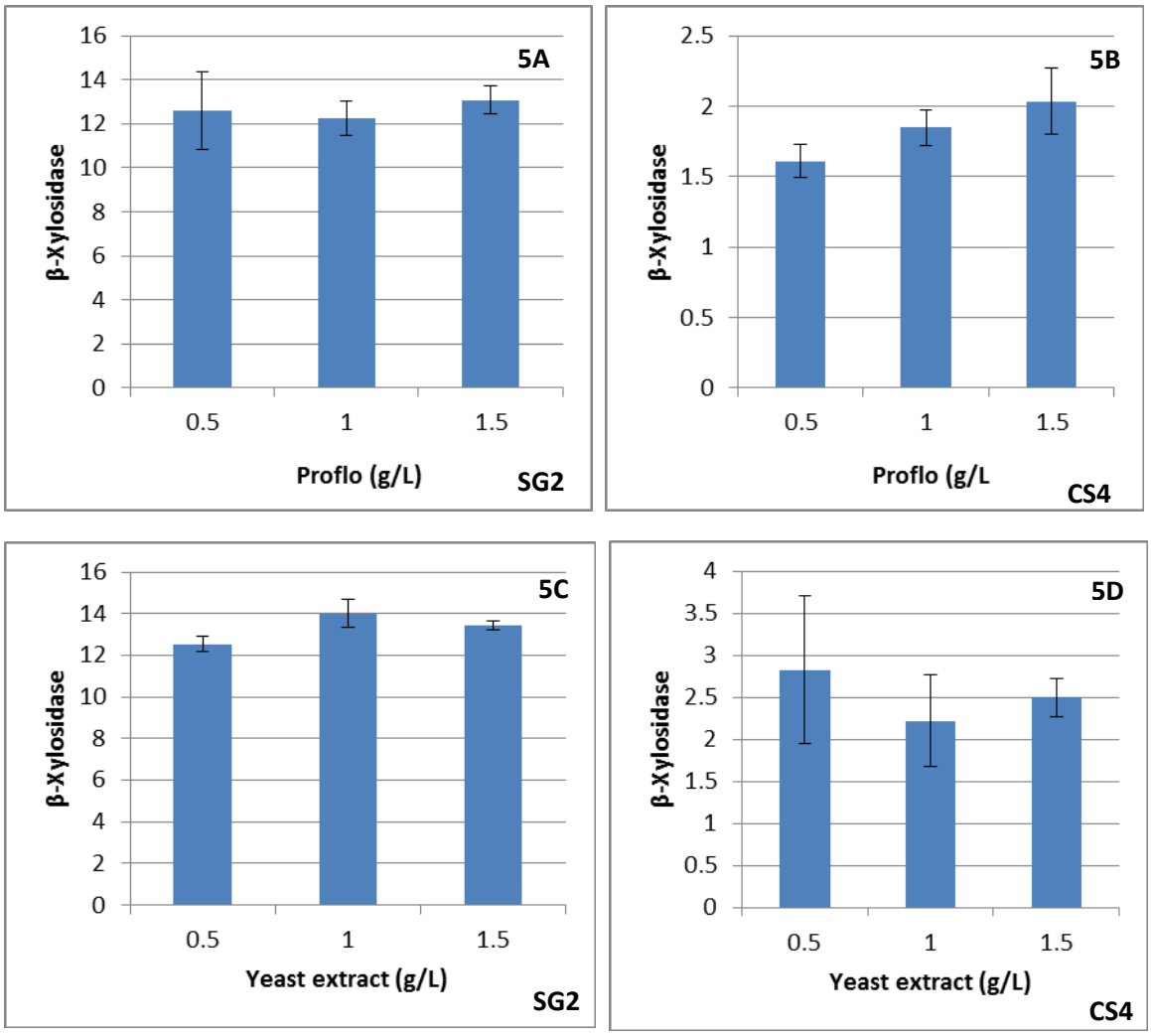

Fig 5A-D: Influence of low concentrations of proflo and yeast extract on production of β-xylosidase of SG2 and CS4.

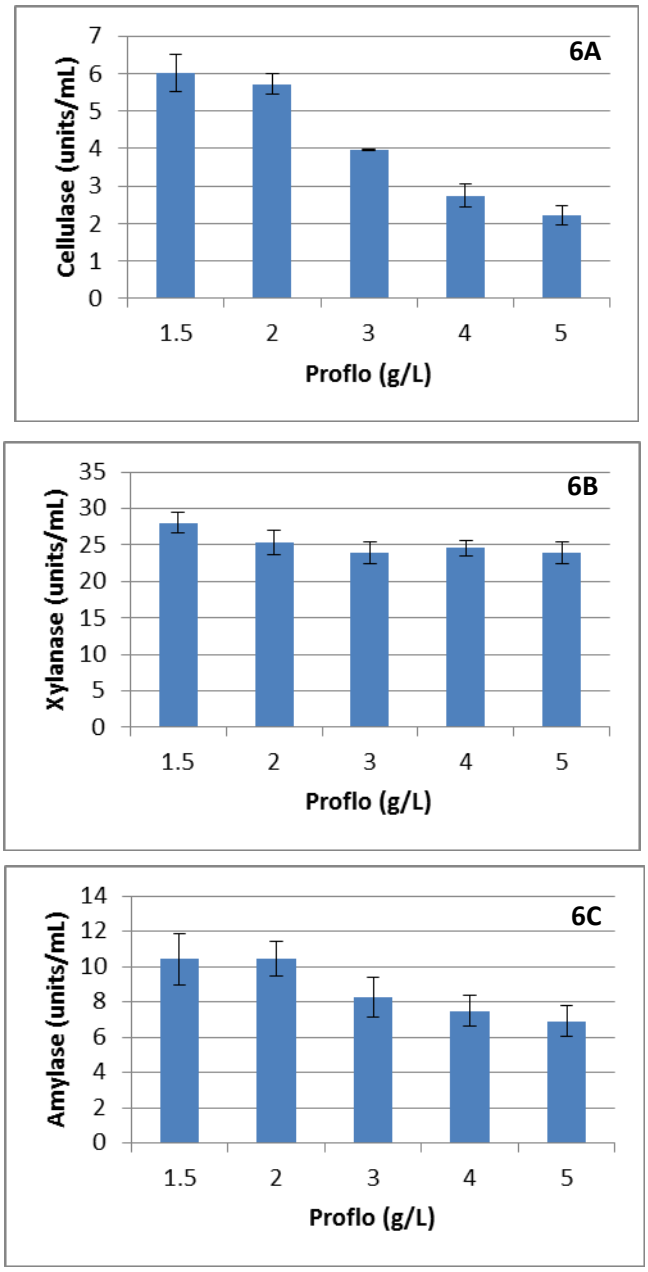

Fig. 6A-C: Influence of high concentrations of proflo on production of cellulase, xylanase and amylase of SG2.

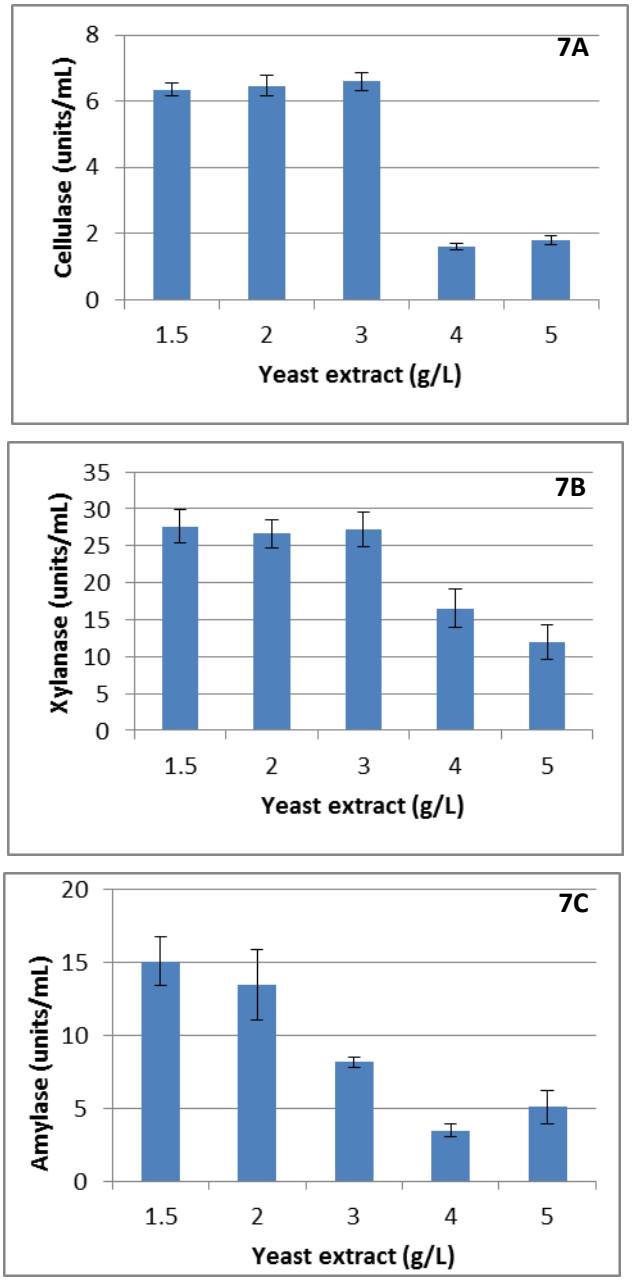

Fig 7A-C: Influence of high concentrations of yeast extract on production of cellulase, xylanase and amylase of SG2.

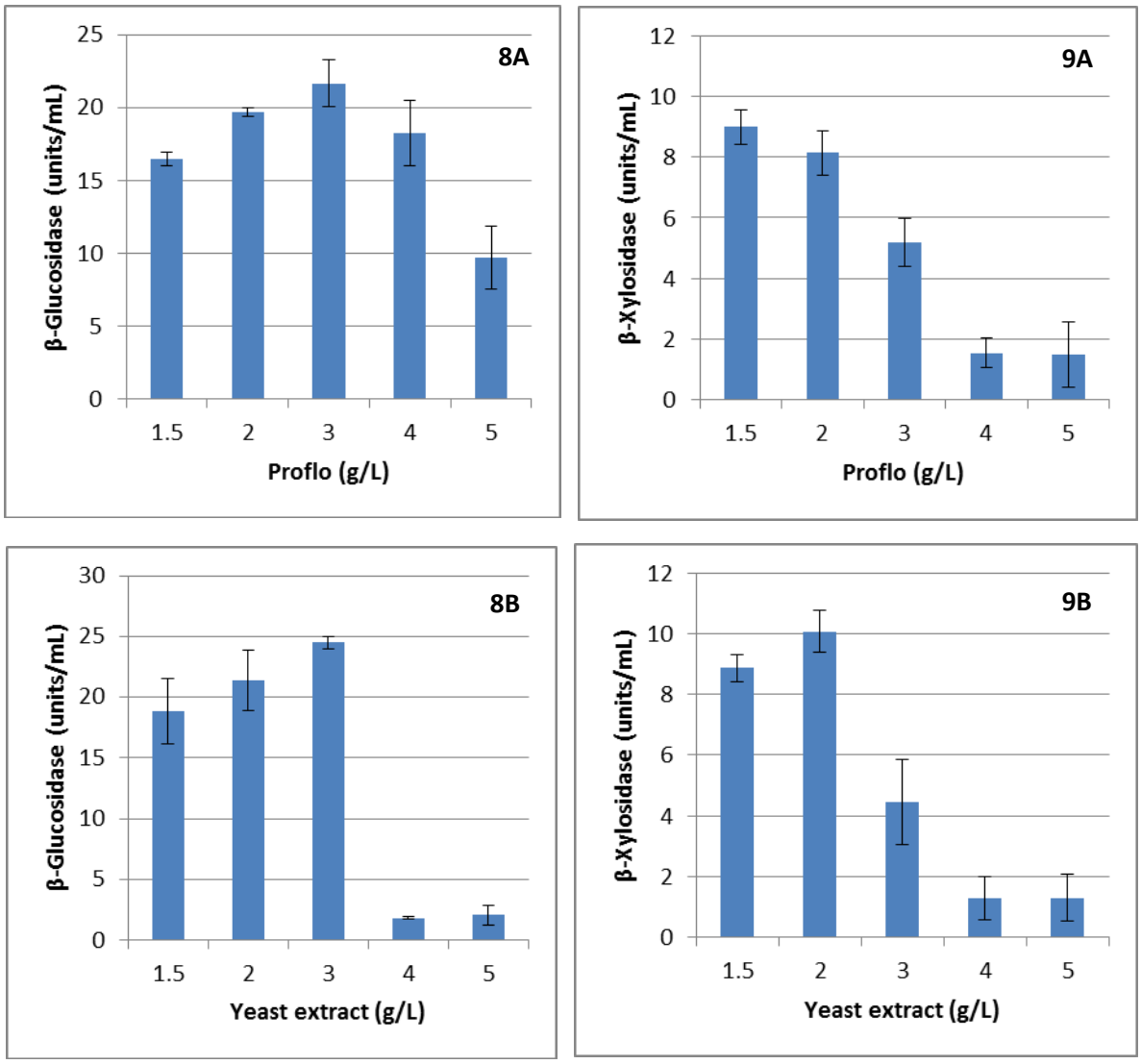

Fig 8 and 9: Influence of high concentrations of proflo and yeast extract on production of β-glucosidase (Fig 8A and B).and β-xylosidase of SG2 (Fig 9A and B).

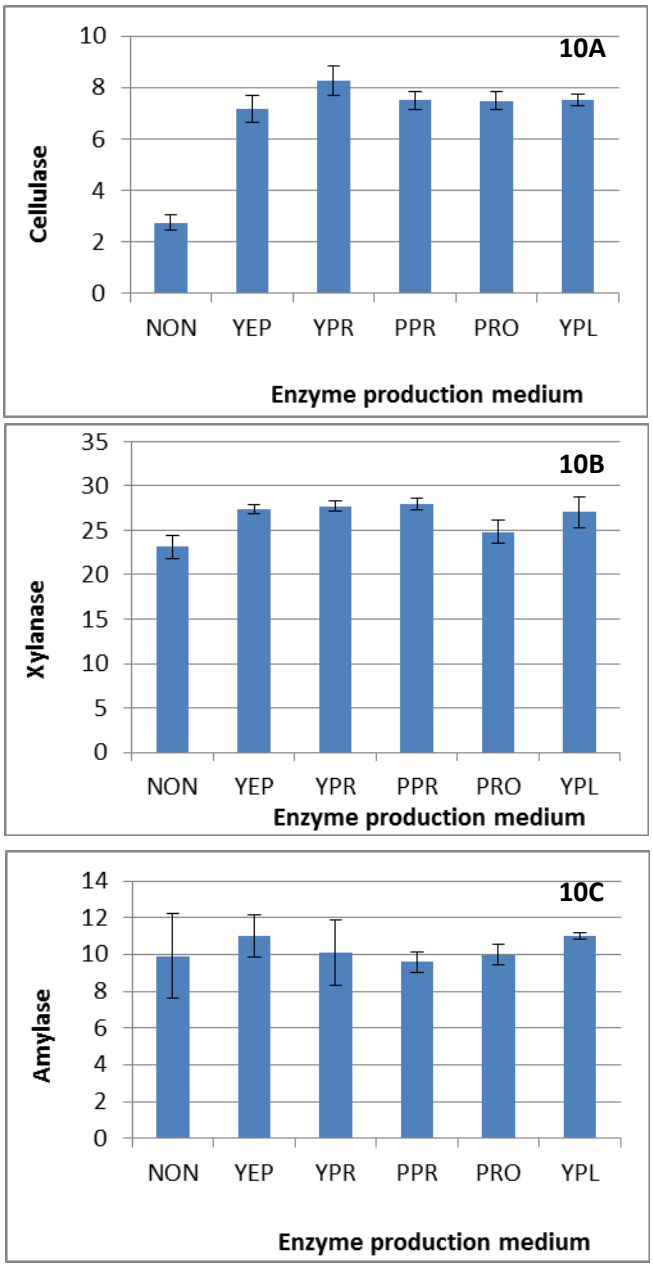

Fig 10A-C: Influence of combination of organic nitrogen sources on production of cellulase, xylanase and amylase of SG2. Each medium consisted of 250 mL of basal medium (BM) to which either of the following were added: 0.5g/L yeast extract and 1.0g/L peptone (**YEP**), 0.5g/L yeast extract and 1.5g/L proflo (**YPR**), 0.5g/L peptone and 1.5g/L proflo (**PPR**), 1.5g/L proflo (**PRO**), 0.5g/L yeast extract, 1.0g/L peptone and lactrol (**YPL**) or the basal medium without organic nitrogen (**NON**).

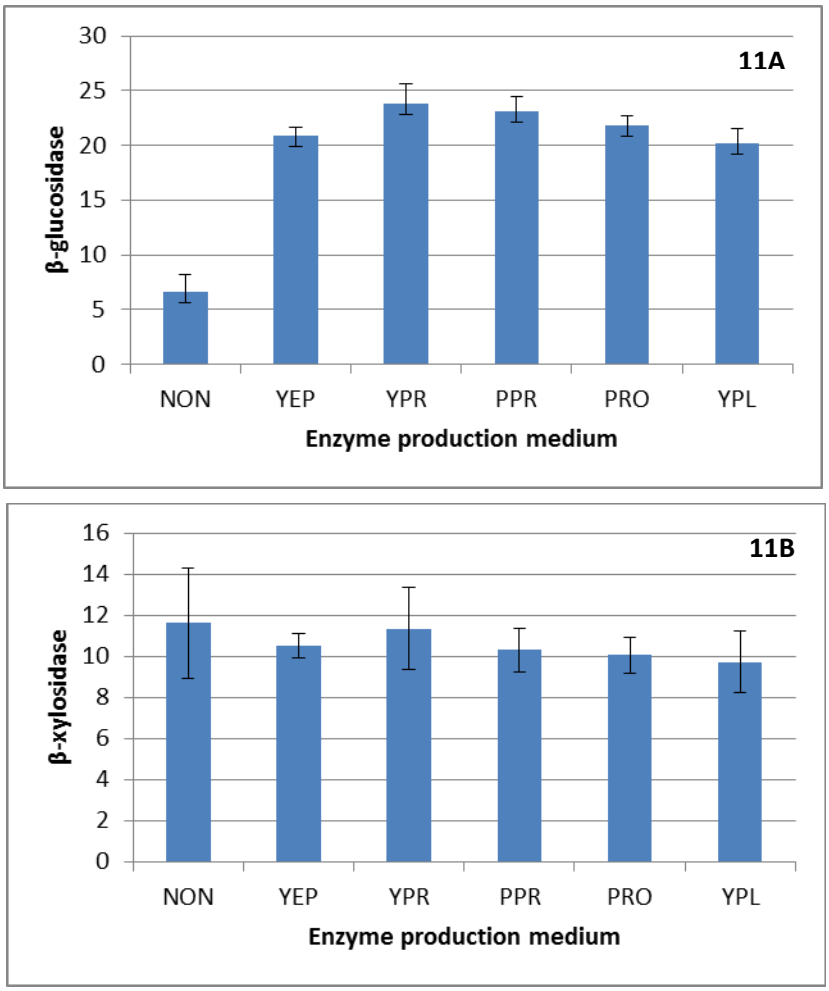

Fig 11A and B: Influence of combination of organic nitrogen sources on production of β-glucosidase and β-xylosidase of SG2. Each medium consisted of 250 mL of basal medium (BM) to which either of the following were added: 0.5g/L yeast extract and 1.0g/L peptone (**YEP**), 0.5g/L yeast extract and 1.5g/L proflo (**YPR**), 0.5g/L peptone and 1.5g/L proflo (**PPR**), 1.5g/L proflo (**PRO**), 0.5g/L yeast extract, 1.0g/L peptone and lactrol (**YPL**) or the basal medium without organic nitrogen (**NON**).

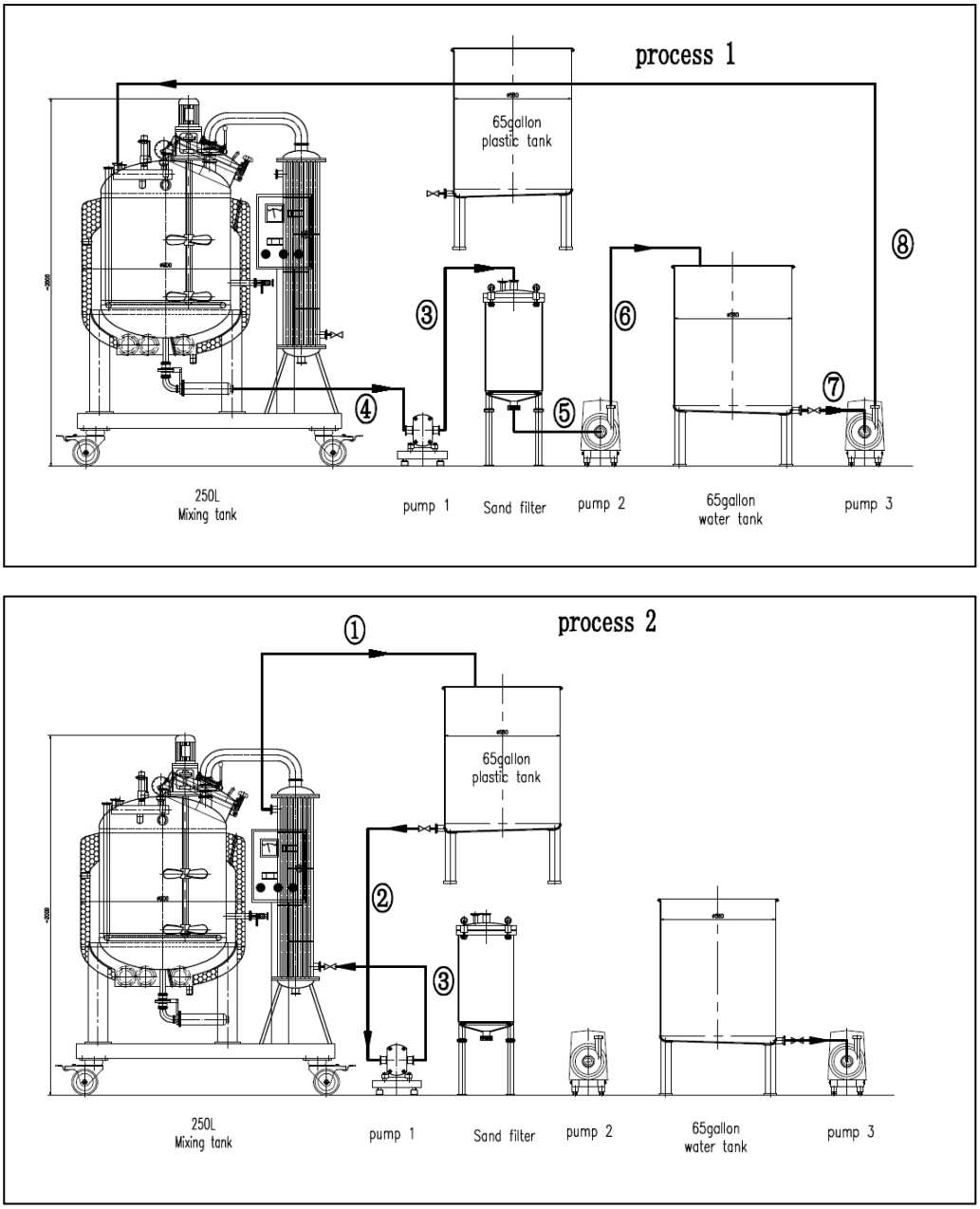

Fig. 12: Process diagram of the bioreactor system for enzyme production, biomass saccharifiaction, fermentation (Process 1) and ethanol extraction (Process 2).

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PART 18 Research Activities of FY-14 (April 1, 2015 to June 30, 2015)
Written by: Benedict Okeke

Introduction and Objectives
In scale-up fermentation, the cost of nutrients is a key factor. Proflo is an inexpensive organic nitrogen source compared to yeast extract. Thus, in the previous quarter, we explored the influence of different concentrations of proflo on enzyme production in comparison with yeast extract. We also examined the effects of combinations of proflo, yeast extract and peptone on enzyme production. In this quarter we focused on completion of remaining aspects of installation of the model farm deployable bioreactor system. We evaluated enzyme production by co-cultures of *Trichoderma* SG2 and a thermotolerant mold CS4 to compose a wider spectrum and more temperature stable mixture of enzymes for biomass hydrolysis. In order to choose the best pretreated biomass for scale up in the bioreactor system, we conducted comparative saccharification of switchgrass pretreated by ammonia fiber explosion (AFEX) and sequential alkali and acid (ALAC) processes; and fermentation of biomass saccharides to ethanol.

Specific Accomplishments

Installation of the bioreactor
Components of the bioreactor system including the Microfueler were installed within the spillage containment concrete curb (approximately 356 cm x 276 cm) built in the last quarter. Air filtration, water deionization and liquid waste disposal systems were installed. Due to safety requirements for operation of the bioreactor system, we began the process for fire alarm installation. We also installed Microfueler for ethanol purification from the bioreactor system.

Biomass Pretreatment
Pulverized switchgrass biomass and shredded waste paper were sequentially pretreated with alkali (1% sodium hydroxide, w/v) and acid (1% sulfuric acid, w/v) hereafter referred to as ALAC pretreatment. In brief, pulverized switchgrass (2kg) was suspended in 9L of 1% NaOH in a plastic autoclave pan and subjected to autoclaving at 121^{o}C (15 psi) for 20 min. Total cycle time from start to end was 60 min. After cooling to room temperature, the NaOH pretreatment liquor was removed by filtering through a white pillow case using Harvest Fiesta Stainless Steel Fruit Press. Pretreated biomass was washed with water until the pH was near neutral. The alkali pretreated biomass was suspended in 9L of 1% sulfuric acid and processed as described for 1% sodium hydroxide; except that it was washed with water until the pH was about 5.0. The ALAC pretreated biomass was placed on aluminum foil and air dried at room temperature. The dry ALAC pretreated biomass was then milled to pass 2 mm mesh using Fritsch Pulverisette 16. The same procedure was employed for the pretreatment of shredded paper except that 2 kg of shredded paper was suspended in 12L of 1% sodium hydroxide w/v or 1% sulfuric acid as needed. ALAC pretreated switchgrass was sieved through 1 mm mesh before saccharification.

Enzyme Production
Enzyme production medium (EPM) was comprised (g per liter) of 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral salts solution (Focht, 1994). Tween 80 was firstly dissolved in a boiled 50 ml aliquot of the medium and then added to the medium. One hundred milliliters of EPM was dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle

size) were added. Flasks were plugged with foam plug and then autoclaved at 121°C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of the thermotolerant fungus (CS4) and *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30°C with orbital shaking (200 rpm) for 6 days. Enzyme (culture supernatant) was recovered by centrifugation (8500 rpm, 20 min) using an Eppendorf centrifuge. SG2 and CS4 inocula were prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30°C for 3 days and then preserved at 4°C.

Enzyme Assays

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs (7 mm in diameter; 10 discs weigh approximately 0.34g) of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2003) and cooled on ice before measuring absorbance at λ405. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol b-D-xyloside. One unit of enzyme activity was 1 µmol of product released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

Comparative Saccharification of AFEX and ALAC Pretreated Biomass

AFEX pretreated and untreated switchgrass and corn stover were obtained from Dr. Venkatesh Balan, Department of Chemical Engineering and Material Science, Michigan State University. ALAC pretreated switchgrass was prepared as described above. The reaction mixture comprised 10 ml of cell-free enzyme in 125 mL screw cap Erlenmeyer flask, 2% of each substrate, 100 µl of 200 mg/L lactrol, 100 µl of 500 mM manganese and 30 µl of 2.5% H_2SO_4 to adjust pH to approximately 5.0. The control was the same as the reaction mixture except that the crude enzyme was replaced with 10.0 mL of distilled water. The reactions were incubated at 50°C in an orbital incubator (80 rpm) for 24 hours. Post incubation, 50 µL of digest was diluted in 950 µl of deionized water and reducing sugar was determined by the DNS method (Miller, 1959).

Comparison of Enzyme Production in Media Amended with Yeast Extract/Peptone (YEP) and Yeast Extract/Proflo (YPR) at Different Concentrations of Mineral Salts.

The full strength mineral salts solution (**FSMSS**) comprised (g per liter) of 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2ml of Fotch mineral elements solution (Focht, 1994). Half strength (**HSMSS**) was a 50% dilution of FSMSS. Both mineral salts solutions were amended with 0.5g/L Tween 80 which was firstly dissolved in an aliquot of the basal mineral salts solution in 50 mL centrifuge tube at 50°C water bath; and then added to the medium. One hundred milliliters of the mineral salts solution containing 0.5g/L yeast extract, 1.0g/L peptone (**YEP**) or 0.5g/L yeast extract, 1.5g/L proflo (**YPR**); were in parallel experiments dispensed into 250 mL Erlenmeyer flasks to which 0.5 g of pulverized switchgrass (< 2 mm particle size) and 0.5 g of paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug and then autoclaved at 121°C for 20

min. After cooling to room temperature, flasks were inoculated with 2 PDA culture discs of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 6 days. **YE** enzyme was produced in **HSMSS**. Enzyme (culture supernatant) was recovered by centrifugation (7500 rpm, 20 min) using an Eppendorf centrifuge. SG2 inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Comparison of Switchgrass Saccharification by Enzymes Produced in Different Media

Saccharification of substrates

Switchgrass was sequentially pretreated with alkali (1% sodium hydroxide and 1% sulfuric acid (**ALAC** procedure) as described above. Enzymes from four media **YEP-A** produced using FSMSS, **YEP-B** produced using HSMSS, **YPR-A** produced using FSMSS, **YPR-B** produced using HSMSS and **YE-A** produced using FSMSS, were examined. The reaction mixture was comprised of 80 ml of cell-free enzymes in 125 mL screw cap Erlenmeyer flask, 2.5% of pretreated switchgrass and 2.5% paper powder, 800 µl of 200 mg/L lactrol, 80 µl of 500 mM Manganese. The reactions were incubated at 50^{o}C in an orbital incubator (80 rpm) for 44 hours. Post incubation, 50 µL of digest was diluted in 950 µL of deionized water and reducing sugar was determined by the DNS method (Miller, 1959).

Ethanol Fermentation of Saccharides Produced with Enzymes from Different Media

This was examined as follows. Replicates of saccharides from each medium were pooled together and 100 mL of each was placed in 125 mL graduated polypropylene bottles. Each saccharide solution was pitched with 0.5 ml of *Saccharomyces cerevisiae* suspension in water (average OD of 50 times dilution of the yeast suspension was approximately 1.08); and 500 µl of lactrol was added from a 200 mg/L stock. Fermentation was carried out at room temperature approximately 20^{o}C for 48 hours and then refrigerated for 48h prior to analysis of ethanol content. Thereafter the samples were further fermented at room temperature for 72h (total fermentation time = 96h) and then subjected to ethanol analysis. Ethanol concentration was assessed using R-Biopharm ethanol analysis kit (catalog number 10 176 290 035), according to the manufacturer's instructions with slight modification. The "reaction mixture" was prepared by dissolving each tablet from bottle 2 in 3 ml of buffer solution from bottle 1. Samples were diluted 100 times by mixing 0.5 ml aliquot of fermented sample with 49.5 ml deionized water in tightly capped 50 ml centrifuge tubes. Each sample reaction in semi micro cuvettes correspondingly received 1.5 ml or 750 µl of reaction mixture, 50 µl or 25 µl of sample. Each blank reaction in semi micro cuvette received 1.5 ml or 750 µl of reaction mixture, 50 µl or 25 µl of deionized water. After 3 minutes, initial absorbance (A1) was read at 340 nm in the order of addition. Thereafter, the reaction was started by adding 25 µl or 12.5 µl of solution 3 (alcohol dehydrogenase; ADH). Final absorbance (A2) for each reaction was then read at 340nm after standing for 5-10 minutes at room temperature; in the order of addition. Ethanol content (g/l) was calculated as follows: Ethanol (c) = [0.7256/ ε] x ΔA; where $\Delta A = (A_2\text{sample}-A_1\text{sample}) - (A_2\text{blank}-A_1\text{blank})$ and ε at 340 nM = 6.3

Production of Cellulolytic-Xylanolytic Enzymes by Co-Cultures of SG2 and CS4

Fifty milliliters of EPM were in parallel experiments dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle size) were added. Flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2 or the thermotolerant fungus (CS4) or both, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm)

for 6 days. Enzyme (culture supernatant) was recovered by centrifugation (7500 rpm, 20 min) using an Eppendorf centrifuge.

Results

The Bioreactor System

Highlights of installation of the farm deployable bioreactor system are presented in Figure 1.

Comparative Saccharification of AFEX and ALAC Pretreated Biomass

Saccharification of AFEX pretreated and untreated switchgrass and corn stover compared to ALAC pretreated and untreated switchgrass are presented in Figure 2. The highest amount of reducing sugar (15.18±0.39g/L) was released from AFEX pretreated corn stover. Sugar released from ALAC pretreated switchgrass was slightly higher than sugar released from AFEX pretreated switchgrass. Saccharification of the pretreated substrates resulted to greater than 90% increase in reducing sugar released compared to the untreated controls (Figure 2). For the untreated substrates, the highest amount of reducing sugar (1.36±0.03) was released from corn stover.

Comparison of Enzyme Production in Media Amended with Yeast Extract/Peptone (YEP) and Yeast Extract/Proflo (YPR) at Different Concentrations of Mineral Salts.

Production of enzymes in selected media amended with yeast extract/peptone (YEP) and yeast extract/proflo (YPR) at different concentrations of mineral salts are presented in Figure 3A-E. In general, dilution of mineral salts to 50% (YEP-B and YPR-B) resulted to only slight differences in cellulase, xylanase and amylase activities. Cellulase activity was less in YPR-B compared to YPR-A. Xylanase activity was slightly higher in YPR-B. Diluted YEP (YEP-B), however, strongly decreased β-glucosidase and β-xylosidase activities. Production β-glucosidase was significantly less in undiluted YPR (YPR-A) compared to diluted YPR (YPR-B). β-xylosidase activity was slightly higher in undiluted YPR (YPR-A), compared to diluted YPR (YPR-B). Average final pH of cultures were 6.2, 6.4, 5.7and 7.2 for YEP-A, YEP-B, YPR-A and YPR-B, respectively. YPR-B was not selected for further studies because of the pH increase to about 7.2; which calls for pH adjustment during fermentation or use of high strength buffer.

Comparison of Biomass Saccharification by Enzymes Produced in Different Media and Fermentation of Saccharides to Ethanol

Saccharification of 2.5% ALAC pretreated switchgrass and 2.5% ALAC pretreated shredded paper powder by enzymes produced in different media is presented in Figure 4. Enzymes made in YEP-A and YE-A produced the highest amount of reducing sugar, 15.03±1.82 g/L and 16.28±1.19 g/L, respectively. The lowest amount of reducing sugar was recorded with enzymes produced in diluted YEP (YEP-B). Ethanol production profiles (Figure 5) from the saccharides produced by the four enzymes were similar to their saccharification profiles (Figure 4). Saccharides produced by YEP-A and YE-A enzymes displayed the highest amount of ethanol after 48 hours and 96 hours of fermentation (Figure 5).

Production of Cellulolytic-Xylanolytic-Amylolytic Enzymes by Mixed Cultures of SG2 and CS4

Co-production of cellulolytic-xylanolytic enzymes by mixed cultures of SG2 and the thermotolerant isolate CS4 are presented in Figure 6A-E. Co-production of the enzymes by mixed cultures of SG2 and CS4 enzymes was expected to improve temperature stability of the

mixtures of enzymes. However, co-production of the enzymes in mixed cultures caused drastic decrease of β-glucosidase and β-xylosidase production compared to monoculture of SG2.

Comparison of biomass saccharification at high temperature by SG2 and CS4

Biomass saccharification at high temperature, at 50°C, 55°C and 60°C by SG-2 and CS4 is shown in Figure 7. SG2 displayed the highest sugar yield at 50°C. Relative to the yield at 50°C (as 100%), 95.95% and 79.07% of the optimum yield were observed at 55°C and 60°C, respectively, for SG2. With CS4, 93.65% and 93.04% of sugar yield were observed at 50°C and 60°C, relative to the yield at the optimum temperature (55°C), as 100%.

Figure 1: Highlights of installation of the farm deployable bioreactor system. Bioreactor (250 liters) for enzyme production, biomass treatment, saccharification, fermentation and ethanol recovery (A); Distillation condenser (B); Sand filter for separation of biomass and solids (C); Storage tank (D); Filter system for air purification (E); Water purification (F); and Microfueller for ethanol purification (G).

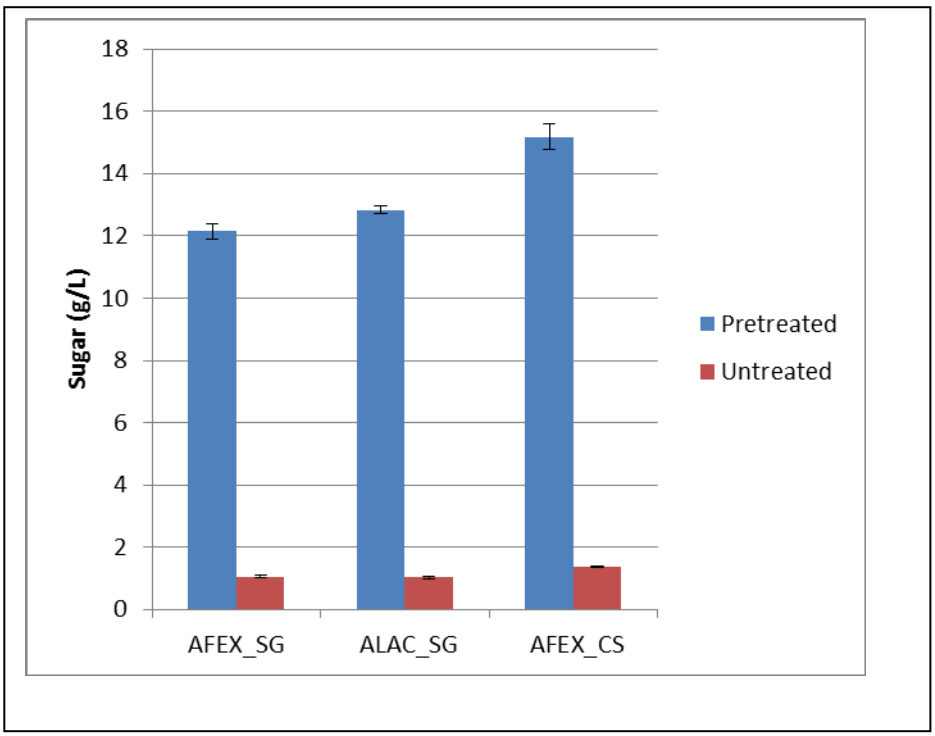

Figure 2: Comparative saccharification of AFEX and ALAC pretreated biomass (2%, w/v) by *Trichoderma* sp. SG2 enzyme. AFEX pretreated switchgrass (AFEX_SG), ALAC pretreated switchgrass (ALAC_SG) and AFEX pretreated corn stover (AFEX_CS). Sugar released is the total reducing sugar measured by the DNS method. Ammonia fiber explosion (AFEX) and alkali-acid (ALAC) pretreatments.

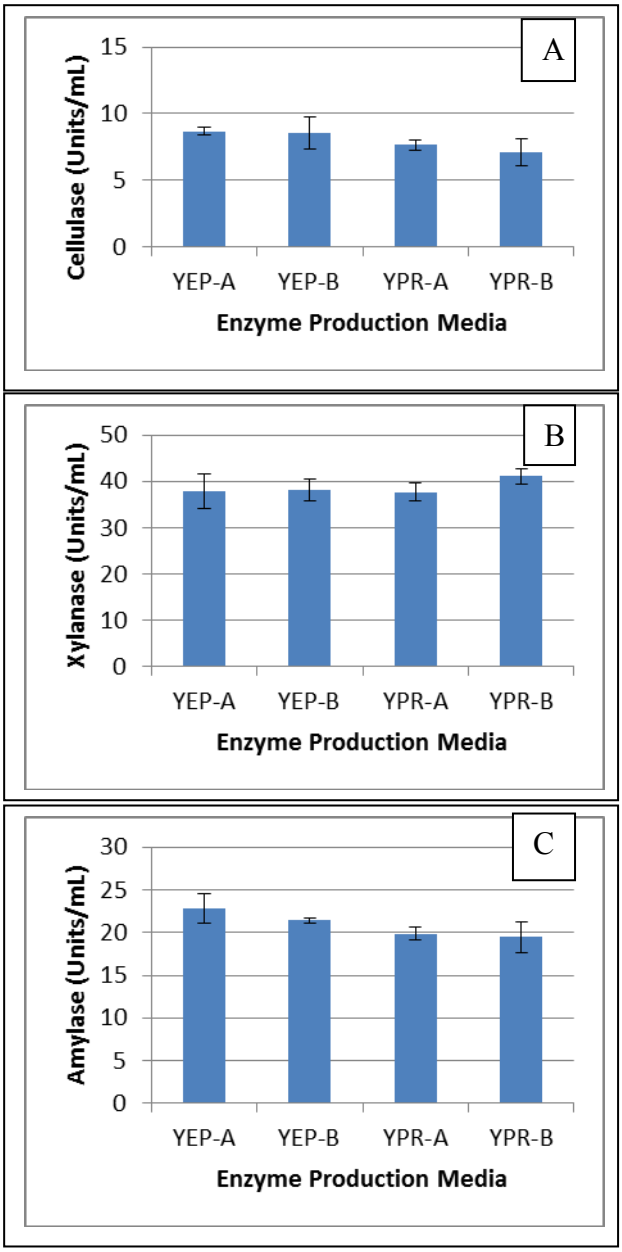

Figure 3A-C: Comparison of cellulase, xylanase and amylase production in media amended with yeast extract/peptone (YEP) and yeast extract/proflo (YPR) at full strength (-A) and half strength (-B) mineral salts concentration.

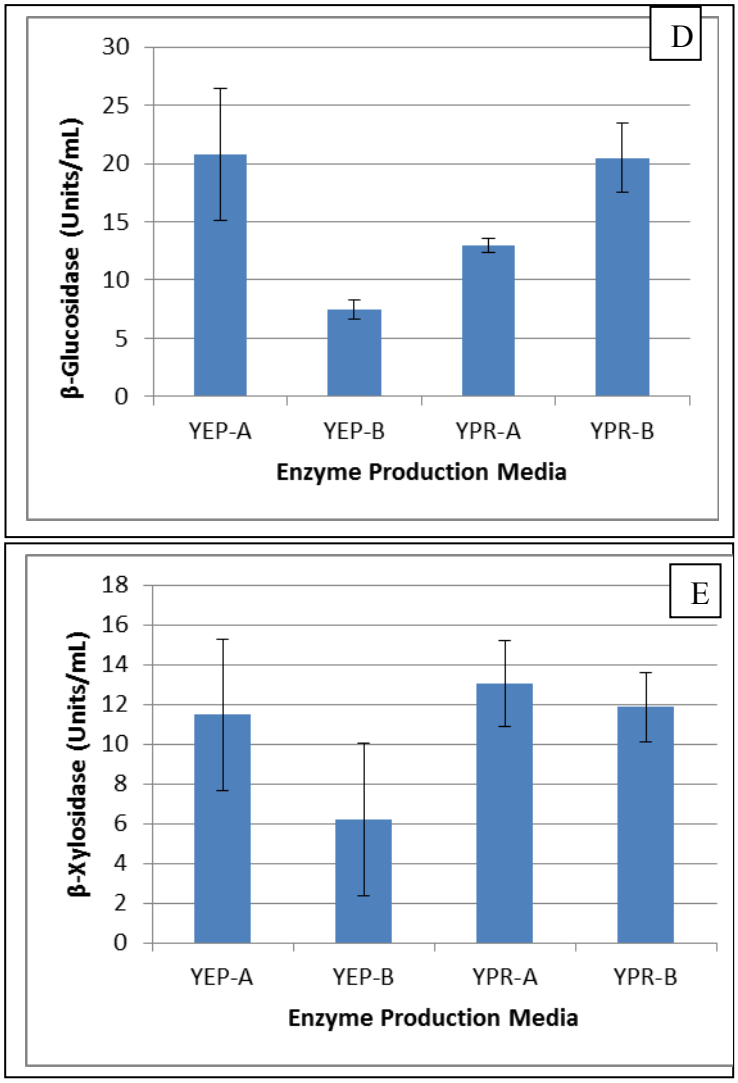

Figure 3D and E: Comparison of β-glucosidase and β-xylosidase production in media amended with yeast extract/peptone (YEP) and yeast extract/proflo (YPR) at full strength (-A) and half strength (-B) mineral salts concentration.

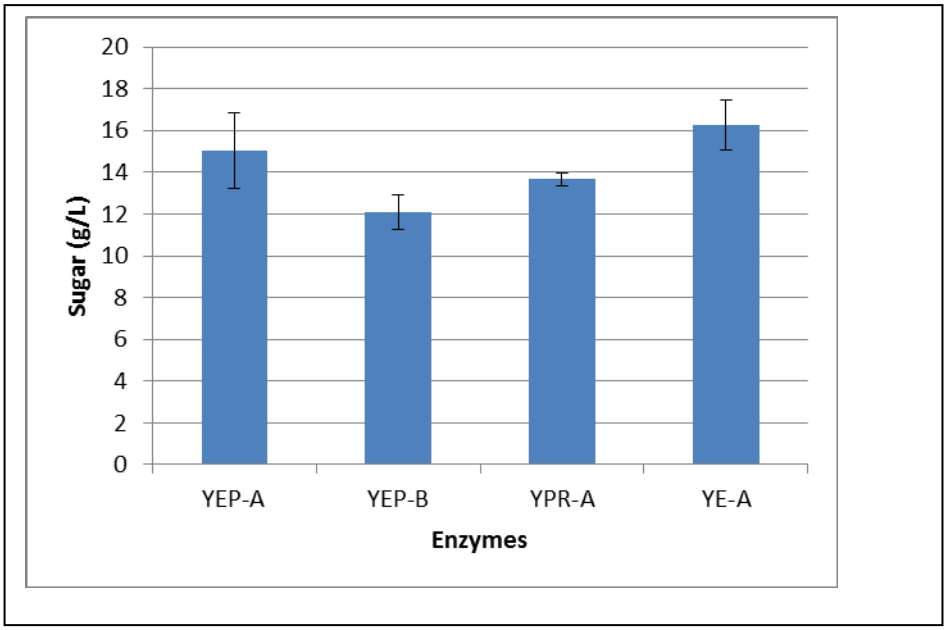

Figure 4: Comparison of ALAC pretreated switchgrass (2.5%, w/v) and paper powder (2.5%) saccharification by enzymes produced in different media; yeast extract/peptone (YEP) and yeast extract/proflo (YPR) at full strength (-A) and half strength (-B) mineral salts concentration. Sugar released is the total reducing sugar measured by the DNS method.

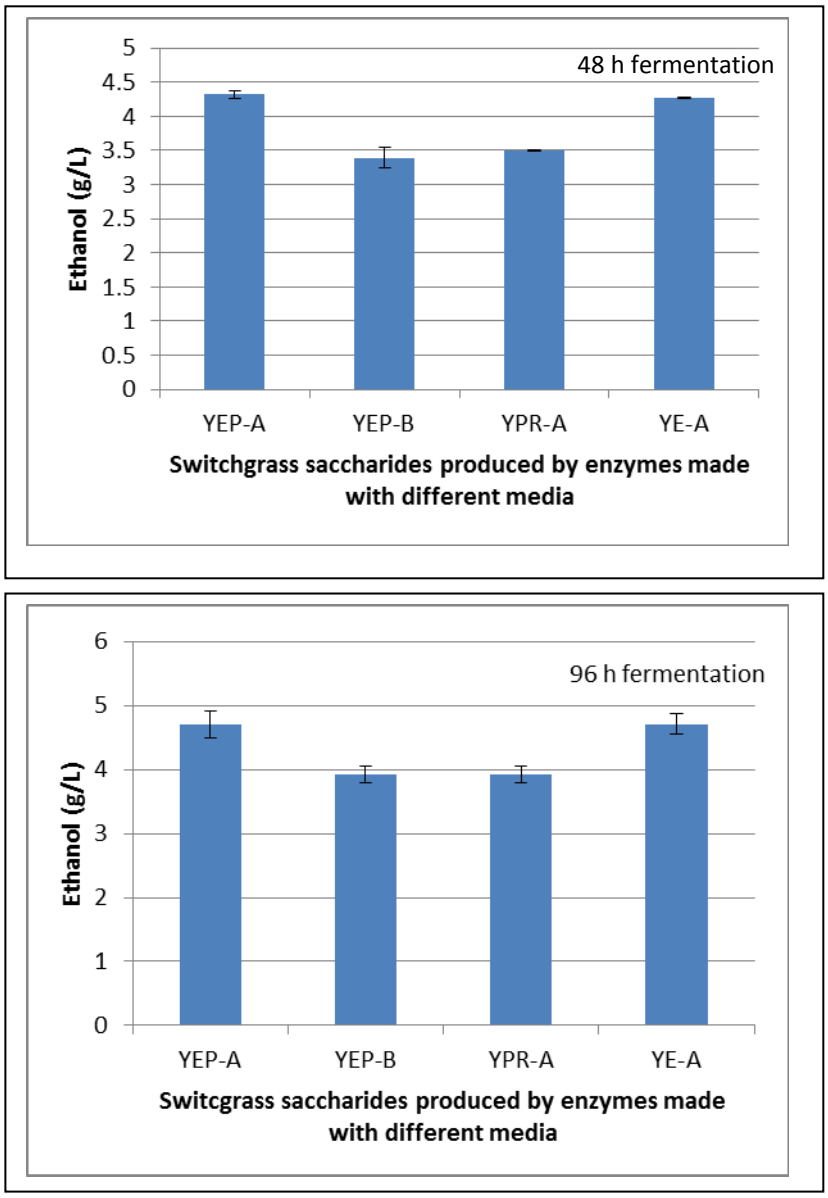

Figure 5: Fermentation to ethanol of saccharides produced from ALAC pretreated switchgrass (2.5%, w/v) and paper powder (2.5%, w/v) by enzymes made in different media; yeast extract/peptone (YEP), yeast extract/proflo (YPR) and yeast extract (YE) at full strength (-A) and half strength (-B) mineral salts concentration.

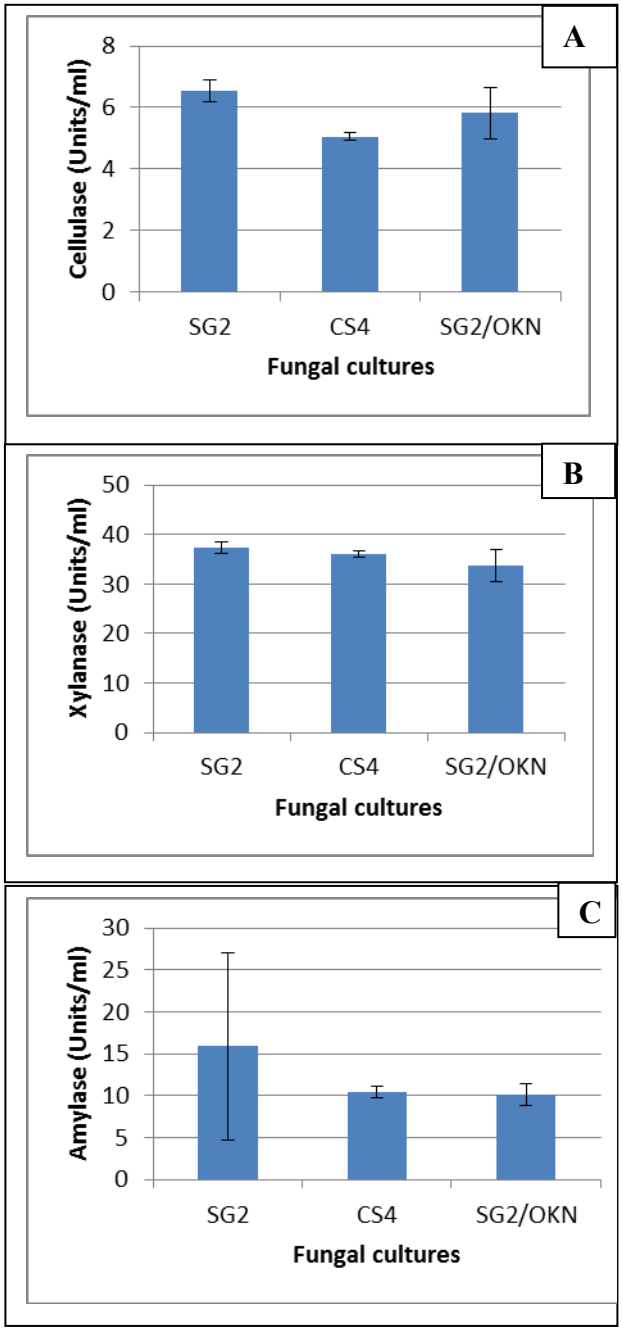

Figure 6A-C: Production of cellulase, xylanase and amylase by mixed cultures of SG2 and CS4.

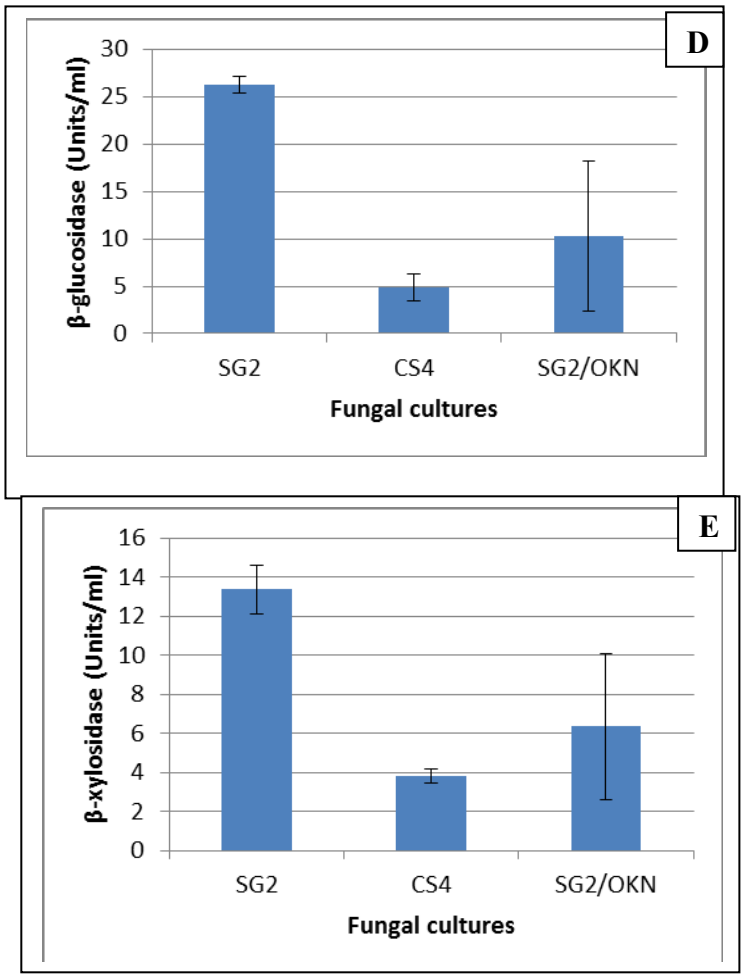

Figure 6D and E: Production of β-glucosidase and β-xylosidase by mixed cultures of SG2 and CS4.

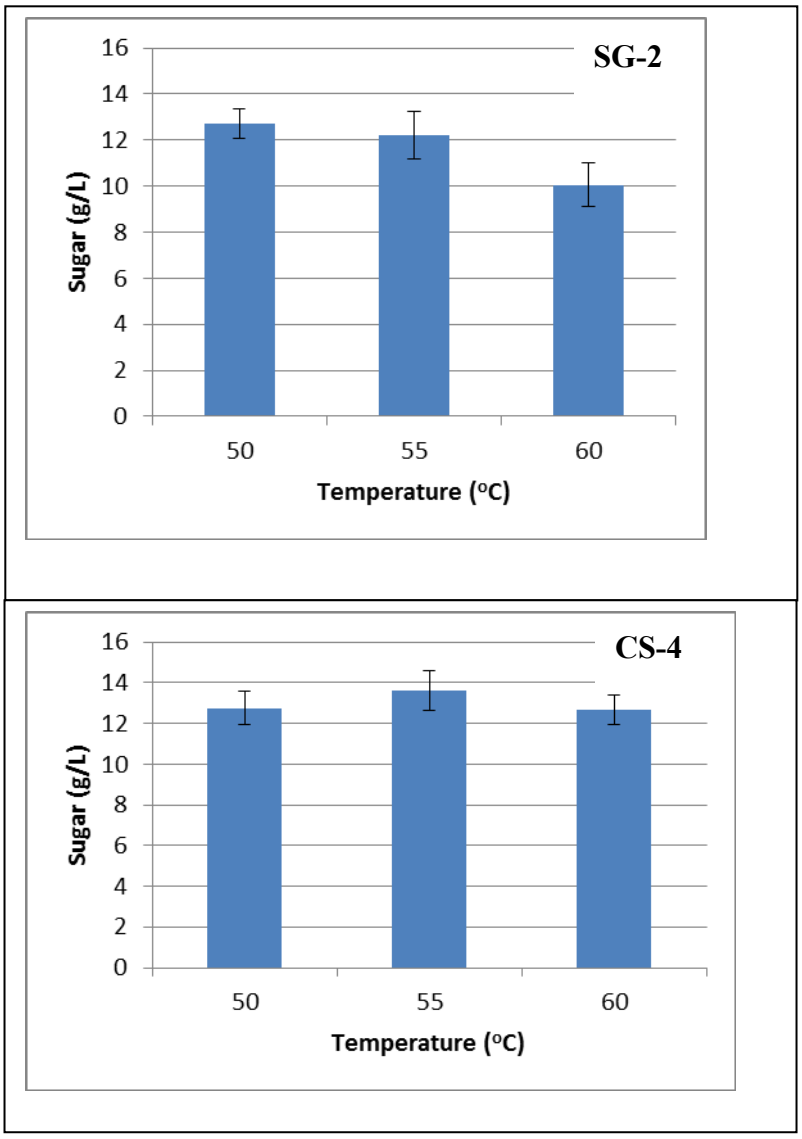

Figure 7: Comparison of biomass saccharification at high temperature by SG2 and CS4.

Publications

1. **Okeke BC**, Hall RW, Nanjundaswamy A, Thomson MS, Deravi Y, Sawyer L, Prescott A. (2015). Selection and molecular characterization of cellulolytic-xylanolytic fungi from surface soil-biomass mixtures from Black Belt sites. *Microbiological Research* 175:24-33.

Presentations

1. Okeke, B.C.; Ingram, C.; Paulk A. and Rafferty, S. (2015). Biomass Conversion by a High Beta-Glucosidase Producing *Trichoderma* species SG2 (Talk delivered by Okeke, BC). The 76th Annual Meeting of the Association of Southeastern Biologists (ASB), Chattanooga TN, April 1–4, 2015.
2. Okeke, B.C.; Ingram, C. and Hardy, B. (2015). Study on Saccharification of Switchgrass and Expired Bread by Fungal Cellulolytic-Xylanolytic-Amylolytic Enzymes. American Society for Microbiology, 114th General Meeting, May 30-June 2, 2014, New Orleans, Louisiana.
3. Ingram, C.; Hardy, B. and Okeke, B.C. (2015). Influence of starch on production of lignocellulolytic and amylolytic enzymes for biomass saccharification. This is Research: Student Symposium, Auburn University, Auburn, AL
4. Ingram, C.; Rodgers, K.; Thomase, P. and Okeke, B.C. (2015). Production of Mixtures of Fungal Amylase, Cellulase and Xylanase in Lignocellulose Medium Amended with Starch. AUM Undergraduate Research Symposium 2015, Montgomery, AL.

Conclusions

Based on production of biomass hydrolyzing enzymes, saccharification and fermentation to ethanol data, YEP-A, and YE-A media are the best for scale up production of biomass ethanol in the bioreactor system. YPR-A media can also be used due to the low cost of Proflo compared to yeast extract and peptone. However, it will require pH adjustment with progress of fermentation or high strength buffer to keep the pH stable. We installed most components of the bioreactor system and scale runs in the bioreactor system will follow.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, WI, pp 407–426.
2. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry* 31: 426-428.
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4. Okeke, B.C. and Lu, J. (2011). Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. *Applied Biochemistry and Biotechnology* 163: 869-881.
5. Saha BC (2003). Purification and properties of an extracellular beta-xylosidase from a newly isolated *Fusarium proliferatum*. *Bioresource Technol*ogy 90:33-38.

PART 19: Research Activities of FY-15 (July 1, 2015 to September 30, 2015).
Written by: Benedict Okeke, Professor, bokeke@aum.edu, 3342443508

Introduction and objectives

This quarter focused on simultaneous saccharification and fermentation of biomass to ethanol using cell-free enzymes (filtered broth), whole-broth biomass saccharification and fermentation to ethanol, biomass pretreatment for scale-up in the bioreactor system, and compliance with safety requirements for operation of the bioreactor system.

Production of biomass hydrolyzing enzymes

Enzyme production medium (EPM) was comprised (g per liter) of 1.0g peptone, 0.5g yeast extract, 0.5g Tween 80, 2g KH_2PO_4, 1.2g $(NH_4)_2SO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 0.003g $FeSO_4.7H_2O$ and 2 ml of Focht mineral element solution (Focht, 1994). Tween 80 was firstly dissolved in a boiled 50 ml aliquot of the medium and then added to the medium. One hundred milliliters of EPM was dispensed into 250 mL Erlenmeyer flasks to which 0.25 g of pulverized switchgrass (< 2 mm particle size) and 0.25 g of paper powder (< 2 mm particle size) were added. Flasks were plugged with foam plug and then autoclaved at 121^{o}C for 20 min. After cooling to room temperature, flasks were inoculated with one PDA culture disc of *Trichoderma* sp. SG2, obtained with sterile 1.25 cm diameter cork borer. Cultures were incubated at 30^{o}C with orbital shaking (200 rpm) for 6 days. Enzyme (culture supernatant) was recovered by centrifugation (8500 rpm, 20 min) using an Eppendorf centrifuge. SG2 inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C.

Assay of enzyme activities

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2003) and cooled on ice before measuring absorbance at λ405. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside. One unit of enzyme activity was 1 µmol of product released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

Pretreatment of switchgrass and shredded waste paper

Switchgrass was pulverized (≤ 2mm) and sequentially pretreated with alkali (1% sodium hydroxide, w/v) and acid (1% sulfuric acid, w/v) hereafter referred to as **ALAC** pretreatment procedure. Pulverized switch grass (2kg) was suspended in 9L of 1% NaOH in a plastic autoclave pan and subjected to autoclaving at 121^{o}C (15 psi) for 20 min. Total cycle time from start to finish was 1 h. After cooling to room temperature, the NaOH pretreatment liquor was removed by filtering through a filter cloth (white pillow) cloth using Harvest Fiesta Stainless Steel Fruit Press. Pretreated biomass was washed with water using the filter cloth

and press until the pH was near neutral. The alkali pretreated biomass was suspended in 9L of 1% sulfuric acid and processed as described for 1% sodium hydroxide; except that it was washed with water until the pH was about 5.0. The ALAC pretreated biomass was placed on aluminum foil and air dried at room temperature. The dry ALAC pretreated biomass was then milled to pass 2 mm mesh using Fritsch Pulverisette 16. The same procedure was employed for the pretreatment of shredded paper except that 2 kg of shredded paper powder was suspended in 12L of 1% sodium hydroxide w/v or 1% sulfuric acid as described. ALAC pretreated switch grass was sieved through 1 mm mesh before saccharification.

Simultaneous saccharification and fermentation to ethanol, with and without pre-saccharification at 50^{o}C and 30^{o}C.

The enzyme was filtered through 0.45 µm membrane filter. The saccharification reaction mixture comprised 60 ml of cell-free enzyme in 125 mL graduated polypropylene bottles, 3g of pretreated switchgrass (5% final concentration), 1200 µl of 200 mg/L lactrol, 600 µl of 500 mM manganese (5mM final concentration) and 180 µl of 2.5% sulfuric acid to adjust the pH to approximately 5.0. Previous studies revealed that maximum conversion of biomass to sugar occurred at 50^{o}C. Hence, the reaction bottles were tightly closed with screw cap and incubated at 50^{o}C in an orbital incubator (80 rpm) for 24 hours. This was followed by pitching with 1g of super start distillers yeast and 1 mL of *Saccharomyces cerevisiae* suspension (OD_{600} of 300 times dilution = 0.855). The reaction was mixed and incubated at 30^{o}C for 6 days for ethanol fermentation (SSF_{24}). In a parallel experiment the reaction mixture was incubated at 30^{o}C for 7 days (SSF_{o}) without pre-saccharifcation at 50^{o}C. In another experiment, SSF_{24} pre-saccharification was conducted at 30^{o}C for 24 hours before pitching and compared to a parallel experiment in which simultaneous saccharification and fermentation were initiated 30^{o}C without pre-saccharifcation (SSF_{o}). Each reaction was charged with 2g of super start distillers yeast. Post incubation, sugar was determined by the DNS method (Miller, 1959).

Ethanol Analysis

Ethanol concentration was assessed using R-Biopharm ethanol analysis kit (catalog number 10 176 290 035), according to the manufacturer's instructions with slight modification. The "reaction mixture" was prepared by dissolving each tablet from kit bottle 2 in 3 ml of buffer solution of kit bottle 1. Samples were clarified by centrifugation at 2500 rpm for 5 minutes, at room temperature. Samples were then and diluted 100 times by mixing 0.5 ml aliquot of fermented sample to 49.5 ml deionized water in tightly capped 50 ml centrifuge tubes. Each sample reaction in semi micro cuvettes received 1.5 ml or 750 ul of reaction mixture, 50 µl or 25 µl of sample. Each blank reaction in semi micro cuvettes received 1.5 ml or 750 ul of reaction mixture, 50 µl or 25 µl of deionized water. After 3 minutes, initial absorbance (A1) was read at 340 nm in the order of addition. Thereafter, the reaction was started by adding 25 µl or 12.5 µl of solution 3 (alcohol dehydrogenase (ADH) approximately 7000U). Final absorbance (A2) for each reaction was then read at 340nm after standing for 5-10 minutes at room temperature; in the order of addition. Ethanol content (g/l) was calculated as follows: Ethanol (c) = [0.7256/ ε] x ΔA; where ΔA = (A_2sample-A_1sample) - (A_2blank-A_1blank) and ε at 340 nm = 6.3.

Evaluation of simultaneous saccharification and fermentation at low temperature

Previous studies revealed that maximum conversion of biomass to sugar occurred at 50°C. However, pre-saccharification at 50°C prior to fermentation was less effective than pre-saccharification at 30°C. This indicated that the biomass hydrolyzing enzymes were less stable at 50°C over a long incubation time. Previously, the effect of biomass saccharification temperature was studied at 35°C to 60°C. Thus biomass saccharification at lower temperature (35°C, 30°C, 25°C) was examined. The enzyme was filtered through 0.45 µm membrane filter. The saccharification reaction mixture comprised 5 ml of cell-free enzyme in 50 mL centrifuge tubes, 0.1g of pretreated switchgrass (2% final concentration), 200 µl of 200 mg/L lactrol, 50 µl of 500 mM manganese (5mM final concentration) and 15 µl of 2.5% sulfuric acid to adjust the pH to approximately 5. The reaction vials were tightly closed with screw cap and incubated at 35°C, 30°C, 25°C and room temperature (approximately 20°C). After 16 hours the reaction was mixed manually. After 24 h saccharification the reaction was spun down (3000 rpm, 15 min, 20°C). Fifty microliters of the clarified upper layer was removed for sugar analysis by DNS method. Super start yeast granules (0.2g) were then added to the saccharification reaction and re-incubated at 35°C, 30°C, 25°C and room temperature (approximately 20°C) for 5 days.

Evaluation of the effect of saccharification temperature on *Trichoderma* enzyme stability

Results obtained from simultaneous saccharification and fermentation at low temperature necessitated evaluation of stability of the enzymes at low temperature (30°C to 35°C). The enzyme, *Trichoderma* crude culture supernatant, was filtered through 0.25 µm membrane filter. Aliquots (7 mL) of the enzyme were placed at room temperature (~20°C), 25°C, 30°C, 35°C and 40°C, in triplicate. Samples were taken after 24h and 52 hours and subjected to cellulase, xylanase, β-glucosidase and β-xylosidase assays. Enzyme incubated at 50°C was sampled after 24h.

Evaluation of pre-saccharification prior to simultaneous saccharification and fermentation

Initially we examined simultaneous saccharification and fermentation to ethanol, with and without 24-h pre-saccharification at the predetermined optimum temperature (50°C) and 30°C. Results suggested that 24-h pre-saccharification at 30°C prior to simultaneous saccharification and fermentation yielded more ethanol than 24-h pre-saccharification at 50°C. Enzyme stability at different saccharification temperature showed that the enzymes were less stable at 50°C (Tables 1-4). Hence pre-saccharification and fermentation at different temperature were examined. The saccharification reaction mixture comprised 60 ml of cell-free enzyme (filtered through 0.45 µm membrane filter), in 125 mL graduated polypropylene bottles, 3g of pretreated switchgrass (5% final concentration), 1200 µl of 200 mg/L lactrol, 600 µl of 500 mM manganese (5mM final concentration), 0.36g of sodium benzoate, and 180 µl of 2.5% sulfuric acid to adjust the pH to about 5.0. Six replicates of the reaction mixture were incubated at 35°C for 24h. Each reaction mixture was inoculated with 2g of superstat yeast and triplicates were then transferred to 30°C and 25°C for simultaneous saccharification and fermentation to ethanol for 6 days. A second set of 6 reaction mixtures were subjected to 40°C pre-saccharification for 24h, followed by simultaneous saccharification and fermentation at 30°C and 25°C for 6 days.

Whole broth simultaneous saccharification and fermentation and comparison with filtered broth simultaneous saccharification and fermentation

Biomass hydrolyzing enzymes were produced as described above. One hundred milliliter cultures were incubated for six days. ALAC pretreated switchgrass (5g), 1 mL of 200 mg/L lactrol, 0.5 mL of 500 mM MnCl, 0.3 mL of 2.5% H_2SO_4 and 1 g Alcotec Turbo yeast. One hundred microliters from 2.5g/50mL stock solutions of each of organic preservatives (calcium propionate, sodium diacetate and sodium benzoate), were added. Flasks were sealed with corks and S-bubble type airlocks containing 5 ml of water. Flasks were incubated at 30°C, 35°C and 40°C for 4 days.

In a parallel experiment, whole broth simultaneous saccharification and fermentation to ethanol was compared to filtered broth simultaneous saccharification and fermentation to ethanol. The reaction mixtures for whole broth simultaneous saccharification and fermentation were as described above except that 300 µL of water was added in place of the three preservatives and incubated at 35°C. The same procedure was followed for filtered broth simultaneous saccharification and fermentation. The 100 mL enzyme used for each reaction mixture was prepared by filtering through 0.45 µM membrane filter.

Evaluation of whole broth simultaneous saccharification and fermentation of mixtures of switchgrass and waste paper powder

This was examined as described under whole broth simultaneous saccharification and fermentation without addition of the organic preservatives (calcium propionate, sodium diacetate and sodium benzoate). Each set of triplicate reaction mixtures contained either 5% ALAC pretreated switchgrass; 5% of ALAC pretreated waste paper powder; a combination of 5% ALAC pretreated switchgrass and 5% of ALAC pretreated shredded waste paper; a combination of 5% ALAC pretreated switchgrass and 2.5% of ALAC pretreated shredded waste paper; or 10 % ALAC pretreated switchgrass. ALAC pretreated shredded waste paper (10 % ALAC) in reaction mixtures was too thick to mix.

Installation of the bioreactor

We completed safety aspects of installation and operation of the bioreactor system.

Results

Simultaneous saccharification and fermentation using cell-free enzyme (filtered broth)

Temperature profile and ethanol yield during simultaneous saccharification and fermentation of ALAC pretreated switchgrass using cell-free enzyme are depicted in Figures 1 and 2. Simultaneous saccharification and fermentation at 30°C (SSF-0) using cell-free enzyme produced more ethanol than pre-saccharification at the pre-determined optimum saccahrification temperature (50°C) for 24 h, followed by fermentation at 30°C (Figure 1c). However, pre-saccharification at 30°C for 24 h (SSF-24) using cell-free enzyme, followed by fermentation at 30°C produced more ethanol than simultaneous saccharification and fermentation at 30°C (Figure 2c). Thus it appears that the enzymes are less stable at 50°C over prolonged incubation time.

Examination of low temperature saccharification of pretreated switchgrass and simultaneous saccharification and fermentation.

Optimum temperature for saccharification of ALAC pretreated switchgrass was previously determined to be 50°C. This did not favor simultaneous sacchrification and fermentation. Thus saccharification of ALAC pretreated switchgrass was examined at 35°C, 30°C, 25°C and

room temperature (approximately 20°C). Saccharification of ALAC pretreated switchgrass by *Trichoderma* sp. SG2 enzyme decreased with decrease in temperature from 35°C to about 20°C (Figure 3). Relative to 35 °C (as 100% yield), about 91.17%, 84.85%, 71.81% saccharide yield was observed in 24h at 30 °C, 25 °C and 20 °C, respectively. Ethanol yield was higher at 25°C; and comparable at 30°C and 35°C.

Stability of biomass hydrolyzing enzymes of *Trichoderma* SG2 at different temperatures for simultaneous saccharification and fermentation

Thermal stability profiles of cellulase, xylanase, β-glucosidase and β-xylosidase at potential temperatures for simultaneous saccharification and fermentation are presented in Tables 1-4, respectively. Cellulase was generally stable at 20°C-35°C; but slightly less stable at 40 °C as the relative activity decreased to 93.87% after 24 h (Table 1). Xylanase was generally stable for 52 hours at 20 °C-40 °C (Table 2). β-glucosidase was generally stable at 20 °C-35 °C; but significantly less stable at 40 °C as the relative activity decreased to 85.28% after 24 hours and 77.5% in 52 hours (Table 3). β-xylosidase was generally stable at 20 °C-35 °C; but slightly less stable at 40 °C as the relative activity decreased to 89.94% after 52 hours (Table 4).

Effect of pre-saccharification prior to simultaneous saccharification and fermentation

Using cell-free enzyme, pre-saccharification at 40 °C for 24 hours prior to simultaneous saccharification and fermentation was more effective than pre-saccharification at 30 °C for 24 hours (Table 5). Ethanol yield at 25 °C and 30 °C were similar. Results confirm that biomass hydrolyzing enzymes of *Trichoderma* SG2 are less stable at higher temperature as observed with pre-saccharification at 50 °C prior to simultaneous saccharification and fermentation to ethanol.

Whole broth simultaneous saccharification and fermentation and comparison with filtered broth simultaneous saccharification and fermentation

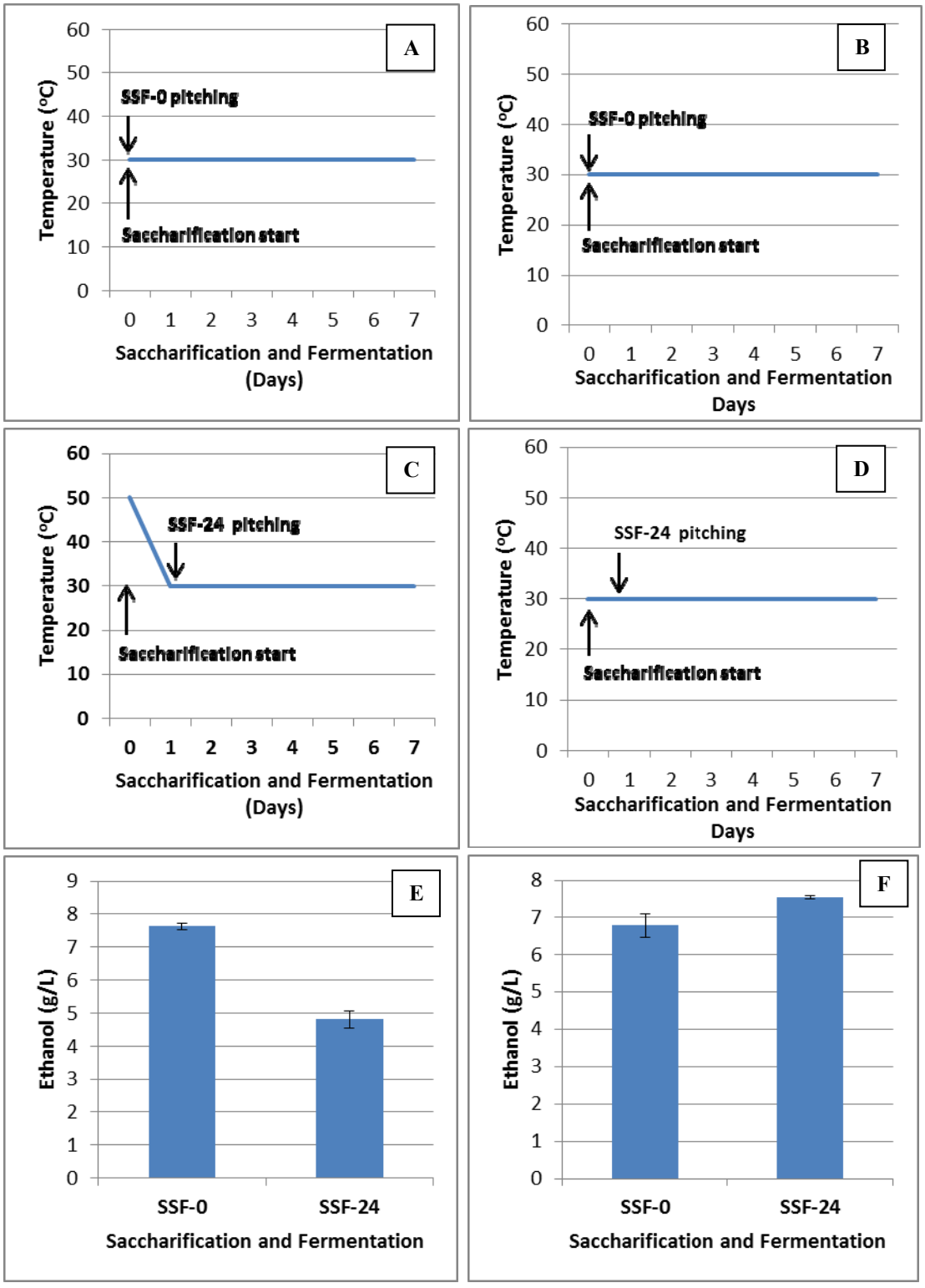
Table 6 presents whole broth simultaneous saccharification and fermentation; and comparison with filtered broth simultaneous saccharification and fermentation. Ethanol yield at 30°C and 35 °C were similar after 4 days. Further incubation for 14 days resulted to only 12.75% and 7.74% increase in ethanol yield compared to the yield after 4 days at 30 °C and 35 °C, respectively. Study conducted at 35°C showed that exclusion of antimicrobial growth preservatives (calcium propionate, sodium benzoate and sodium diacetate) to the reaction mixture did not result to a significant change in ethanol yield. Whole broth pre-saccharification at 40 °C followed by simultaneous saccharification and fermentation at 25 °C produced 4.97 ± 0.18 g/L and 5.47 ± 0.13 g/L ethanol after 4 and 14 days respectively.

Evaluation of whole broth simultaneous saccharification and fermentation of mixtures of switchgrass and shredded waste paper

Whole broth simultaneous saccharification and fermentation of mixtures of switchgrass and waste paper powder is presented in Table 7. The highest ethanol yield was observed with 5% shredded waste paper followed by 10% switchgrass. Ethanol yield observed with combination of 5% switchgrass and 2.5% shredded waste paper was higher than observed with combination of 5% switchgrass and 5% shredded waste paper.

The Bioreactor System

A bioreactor system was developed, constructed and installed. Safety aspects of installation and operation of the bioreactor system were met for scale up of simultaneous saccharification and fermentation of biomass to ethanol.



Figures 1 and 2. Temperature profiles for simultaneous saccharification and fermentation (SSF-0) without pre-saccharification (A and B) and simultaneous saccharification and fermentation with 24 pre-saccharification (SSF-24) at 50°C (C) and 30°C (D). Ethanol levels in simultaneous saccharification and fermentation with 24 hour pre-saccharification (SSF-24) at 50°C (E) and 30°C (F).

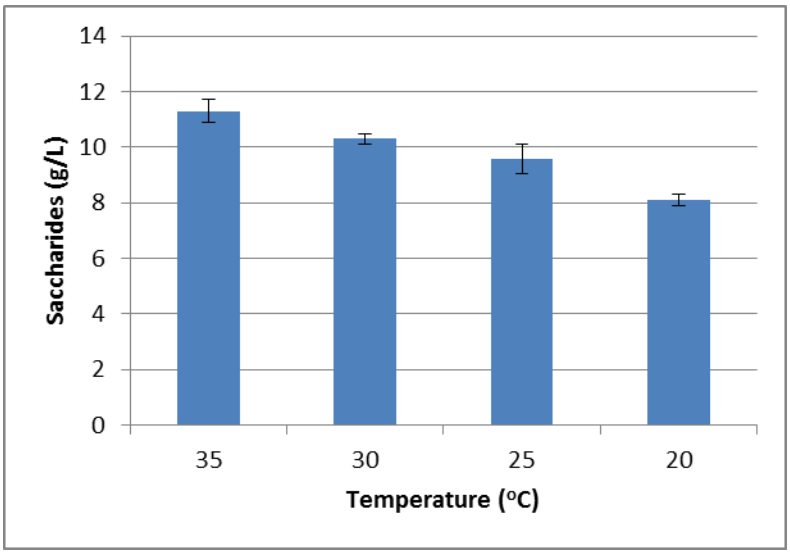


Figure 2. Switchgrass biomass saccharification at low temperature. Incubation at room temperature was approximately 20°C.

Table 1: Temperature stability of cellulase in biomass hydrolyzing enzymes of *Trichoderma* SG2

Temperature (°C)	Cellulase (Units/ml)		Relative activity (%)[a]	
	24 h	52 h	24 h	52 h
20	6.65±0.58	6.64±0.18	96.29	96.04
25	6.74±0.16	6.47±0.22	97.54	93.67
30	6.91±0.61	6.67±0.11	99.94	96.45
35	7.18±0.27	6.98±0.16	103.93	100.94
40	6.49±0.09	6.52±0.27	93.870	94.35

[a] Results were normalized relative to the initial cellulase activity (6.91±0.24 U/mL) as 100%. Activity units are total activity in a 30 min assay.

Table 2: Temperature stability of xylanase in biomass hydrolyzing enzymes *Trichoderma* SG2

Temperature (^{o}C)	Xylanase (Units/ml)		Relative activity (%)[a]	
	24 h	52 h	24 h	52 h
20	45.02±1.91	45.35±2.10	101.57	102.30
25	44.40±0.80	44.92±1.20	100.18	101.33
30	42.55±6.17	44.97±2.25	95.99	101.45
35	45.18±1.21	43.68±1.15	101.93	98.54
40	43.73±1.37	44.70±1.76	98.66	100.84

[a]Results were normalized relative to the initial xylanase activity (44.32±1.94 U/mL) as 100% Activity units are total activity in a 30 min assay.

Table 3: Temperature stability of β-glucosidase in biomass hydrolyzing enzymes *Trichoderma* SG2

Temperature (^{o}C)	β-glucosidase (Units/ml)		Relative activity (%)[a]	
	24 h	52 h	24 h	52 h
20	15.51±0.17	15.78±0.22	99.25	100.95
25	15.25±0.17	15.68±0.18	97.54	100.31
30	15.26± 0.30	15.43±0.36	97.65	98.72
35	14.83±0.13	15.01±0.28	94.88	96.05
40	13.33±0.15	12.10±0.10	85.28	77.40

[a]Results were normalized relative to the initial β-glucosidase activity (15.63±0.32 U/mL) as 100%.. Activity units are total activity in a 30 min assay.

Table 4: Temperature stability of β-xylosidase in biomass hydrolyzing enzymes *Trichoderma* SG2

Temperature (^{o}C)	β-xylosidase (Units/ml)		Relative activity (%)[a]	
	24 h	52 h	24 h	52 h
0	10.76±0.11	11.80±0.15	96.99	106.30
25	11.23±0.21	11.63±0.25	101.20	104.80
30	11.08±0.08	11.71±0.10	99.84	105.55
35	11.01±0.16	11.15±0.10	99.24	100.45
40	10.45±0.12	9.98±0.30	94.14	89.94

[a]Results were normalized relative to the initial β-xylosidase activity (11.10±0.14 U/mL) as 100%

Table 5: Pre-saccharification, and simultaneous saccharification and fermentation at different temperatures.

Pre-saccharification Temperature (^{o}C)	SSF Temperature (^{o}C)	Ethanol (g/L)	SD
35	30	0.406	0.048
35	25	0.397	0.103
40	30	0.477	0.092
40	25	0.483	0.091

SSF: Simultaneous Saccharification-Fermentation;
SD: standard deviation of triplicates

Table 6: Whole Broth simultaneous saccharification and fermentation and comparison with filtered broth simultaneous saccharification and fermentation

Enzymes for Saccharification	**Temperature (^{o}C)**	4 Days		14 Days	
		Ethanol (g/L)	SD	Ethanol (g/L)	SD
Whole broth with organic preservatives	30	5.300	0.180	5.976	0.464
Whole broth with organic preservatives	35	5.421	0.572	5.841	0.471
Whole broth with organic preservatives	40	4.975	0.189	5.475	0.137
Whole broth without organic preservatives	35	5.505	0.811	5.613	0.723
Filtered broth without organic preservatives	35	4.618	0.477	5.268	0.610

.

Table 7: Whole broth simultaneous saccharification and fermentation of mixtures of pretreated switchgrass (SG) and shredded waste paper powder (WP)

Treatments	Ethanol (g/L)	SD
Whole broth culture (100 mL) + 5% SG	5.309	0.186
Whole broth culture (100 mL) + 5% WP	12.062	0.236
Whole broth culture (100 mL) + 5% SG + 2.5% WP	7.758	1.648
Whole broth culture (100 mL) + 10% SG	8.261	1.000
Whole broth culture (100 mL) + 5% SG + 5% WP	7.352	0.212

Presentations

1. Benedict Okeke, Ananda Nanjundaswamy, Rosine Hall and Christiane Ingram (2015). Highlights of Research on Development of Field Deployable Bioreactor Process for Biomass Conversion to Ethanol. *This is Research: Faculty Symposium.* September 30th, Auburn University,https://cws.auburn.edu/OVPR/pm/thisisresearch/faculty.

Conclusions

A bioreactor system for simultaneous saccharification and fermentation; and separate saccharification and fermentation of biomass to ethanol was developed. Studies indicated that whole broth simultaneous saccharification and fermentation at 30 ^{o}C and 35 ^{o}C compared with cell free enzyme (filtered broth) simultaneous saccharification and fermentation is potentially more cost effective for converting biomass to ethanol using *Trichoderma* SG2 enzyme.

References

1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, WI, pp 407–426.
2. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry* 31: 426-428.
3. Okeke B.C (2014). Cellulolytic and xylanolytic potential of high β-glucosidase producing *Trichoderma* from decaying biomass. *Applied Biochemistry and Biotechnology*. 174:1581-98. doi: 10.1007/s12010-014-1121-x.
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PART 20: Research Activities of FY-16 (October 1, 2015 to December 31, 2015).
Written by: Benedict Okeke,

Introduction and objectives

In the previous quarter effort was focused on simultaneous saccharification and fermentation of biomass to ethanol using cell-free enzymes (filtered broth), whole-broth biomass saccharification and fermentation to ethanol and biomass pretreatment for scale-up in the bioreactor system. In whole broth simultaneous saccharification and fermentation of mixtures of pretreated substrates, the highest amount of ethanol 12.06g/L was obtained with 5% pretreated paper powder; whereas 5.309g/L and 8.26 g/L were respectively recorded with 5% and 10% switchgrass in flasks. In this quarter effort was focused on process scale-up in the bioreactor and pretreatment of biomass to obtain sufficient quantity for process scale-up in the bioreactor system.

Scale up of production of biomass hydrolyzing enzymes in the bioreactor system

The bioreactor was cleaned using One Step no rinse cleanser and then rinsed thoroughly with water. Thereafter 200 liters of deionized water, supplied through Elga Labwater Vision250 wall mounted deionization system was added to the bioreactor. Enzyme production medium (EPM) was comprised (g per 200 liters) of 200g peptone, 100g yeast extract, 100g Tween 80, 400g KH_2PO_4, 240g $(NH_4)_2SO_4$, 100g $MgSO_4.7H_2O$, 20g $CaCl_2$, 0.6g $FeSO_4.7H_2O$, 0.4g lactrol and 400 ml of Focht mineral element solution (Focht, 1994). The 100g of Tween 80 was suspended 100 ml of warm water and then added to the medium. Pulverized switchgrass (1000g) and pulverized paper powder (1000g) were pre-sterilized by autoclaving at 121˚C for 20 minutes; and then added to the bioreactor. The enzyme production medium was then sterilized *in situ* in the bioreactor by heating at 108^{o}C for 1 hour. The bioreactor was allowed to cool to room temperature for about 60 hours before inoculation with agar blocks from 50 potato dextrose agar plate cultures of *Trichoderma* sp. SG2. The inoculum was prepared by inoculating the center of potato dextrose agar (PDA) plates with a piece of PDA culture of each isolate and incubated at 30^{o}C for 3 days and then preserved at 4^{o}C. The bioreactor was operated at 30^{o}C with aeration and agitation. Air was supplied using a rotary vane deluxe system GAST oiless 1023 series model #1423-101Q-G626X equipped with 6 air outlets. Four of the air outlets were closed, one was open and one was connected to the bioreactor. Air was filtered using a 10" Emflon PFR 0.2µm sterile air filter (PALL AB1PFR7WH4) and pre-filter (PALL AB1U010Z7H4) installed in a filter housing (PALL FBT011G23H43A). Samples were collected through a sampling valve. The above procedure hereafter referred to as first run was employed for the second run except that peptone, yeast extract and ammonium sulfate were separately dissolved in 4 L of deionized water added and sterilized by autoclaving at 121˚C for 20 minutes. The solution was cooled to room temperature and stored at 4˚C during the 60 hour cooling time for the bioreactor. Thereafter the solution was added to the bioreactor with other medium ingredients.

Assay of enzyme activities

Cellulase activity (filter paper cellulase) and xylanase activities were assayed as previously described (Okeke and Jue, 2010). Briefly, for cellulase, the reaction mixture comprised 10 discs of Whatman #1 filter paper, 0.5 ml of 100 mM sodium acetate buffer pH 5.0, 0.5 ml of enzyme and incubated in a 50°C water bath for 30 min. Xylanase reaction mixture contained 0.01g of xylan in place of filter paper disc. Amylase assay mixture comprised 0.5 ml of 1% soluble starch solution in 100 mM sodium acetate buffer pH 5.0 and 0.5 ml of enzyme (cell-

free culture supernatant). Reducing sugar was determined by the DNS method of Miller, 1959. For β-glucosidase, the reaction mixture comprised 100 µl of enzyme, 800 µl of 100 mM sodium acetate buffer pH 5.0 and 100 µl of 40 mM ρ-nitrophenol β-D-glucoside in 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 minutes in a 50°C water bath (Saha, 2003) and cooled on ice before measuring absorbance at λ405. β-xylosidase activity was determined using the same method except that the substrate was 20 mM ρ-nitrophenol β-D-xyloside. One unit of enzyme activity was 1 µmol of product (reducing sugar) released in the assay mixture in 30 min and expressed per mL of the enzyme preparation.

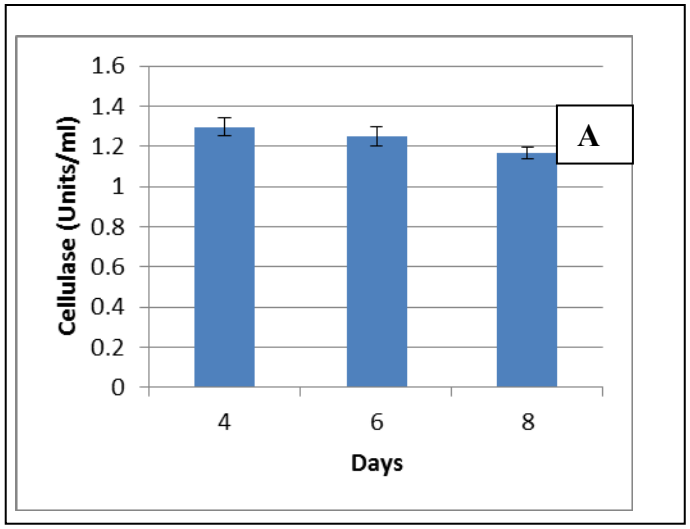
Pretreatment of switchgrass and shredded waste paper

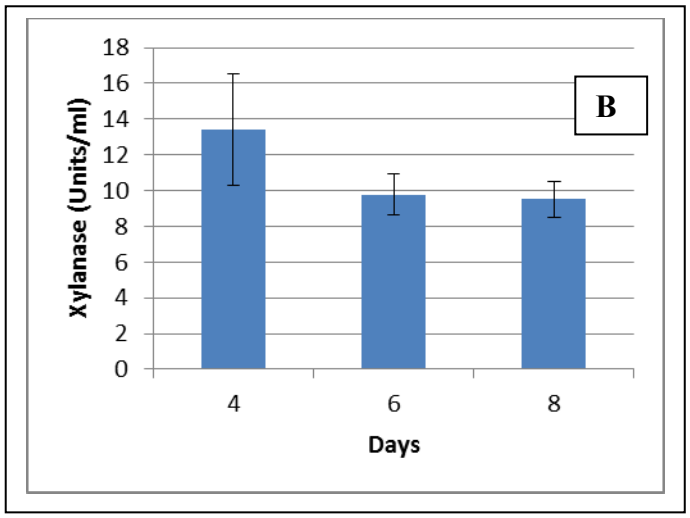
Switchgrass was pulverized (≤ 2mm) and sequentially pretreated with alkali (1% sodium hydroxide, w/v) and acid (1% sulfuric acid, w/v) hereafter referred to as **ALAC** pretreatment procedure. Pulverized switch grass (2kg) was suspended in 9L of 1% NaOH in a plastic autoclave pan and subjected to autoclaving at 121^{o}C (15 psi) for 20 min. Total cycle time from start to finish was 1 h. After cooling to room temperature, the NaOH pretreatment liquor was removed by filtering through a filter cloth (white pillow) cloth using Harvest Fiesta Stainless Steel Fruit Press. Pretreated biomass was washed with water using the filter cloth and press until the pH was near neutral. The alkali pretreated biomass was suspended in 9L of 1% sulfuric acid and processed as described for 1% sodium hydroxide; except that it was washed with water until the pH was about 5.0. The ALAC pretreated biomass was placed on aluminum foil and air dried at room temperature. The dry ALAC pretreated biomass was then milled to pass 2 mm mesh using Fritsch Pulverisette 16. The same procedure was employed for the pretreatment of shredded paper except that 2 kg of shredded paper powder was suspended in 12L of 1% sodium hydroxide w/v or 1% sulfuric acid as described.

Results

Scale up of enzyme production in the bioreactor system

Cellulolytic, xylanolytic and amylolytic activities from the first run of the bioreactor are presented in Figures 1A-1E. Cellulase production reached maximum after 4 days (Fig. 1A). Comparable levels of cellulase were observed on days 6 and 8. Xylanase and amylase activities reached maximum on day4 and thereafter decreased on days 6 and 8 (Fig. 1B-C). β-glucosidase activity reached maximum on day 6 (Fig. 1D). The highest level of β-xylosidase activity was observed on day 8. Figures 2A-E present cellulolytic, xylanolytic and amylolytic enzymes activities from the second run of the bioreactor. The highest production of cellulase was recorded after 4 days (Fig. 2A) and was similar to the activity on day 6. Production of xylanase and amylase reached maximum on day 4 and thereafter decreased on days (Fig. 2B-C). The highest level of β-glucosidase activity was observed on day 6 (Fig. 1D); β-xylosidase activity was similarly optimal on day 6. Results observed in the two runs of the bioreactor were generally similar. In a third run of the bioreactor, cellulase, xylanase, amylase, beta-glucosidase and beta-xylosidase activities were 1.66 ±0.04, 23.59± 3.91, 3.13±0.036; 6.53±0.83; 4.71±0.16, respectively. Whole broth simultaneous saccharification and fermentation of 10% switchgrass in the bioreactor using the third run yielded only about 0.5g ethanol per liter. The levels of cellulolytic, xylanolytic and amylolytic activities observed were much less than previously observed in flask experiments, indicating scale up inefficiency that needs optimization. Thus, the low levels of enzyme activities recorded in the bioreactor are not suitable for downstream steps in the process. Our laboratory will continue research to optimize the process.





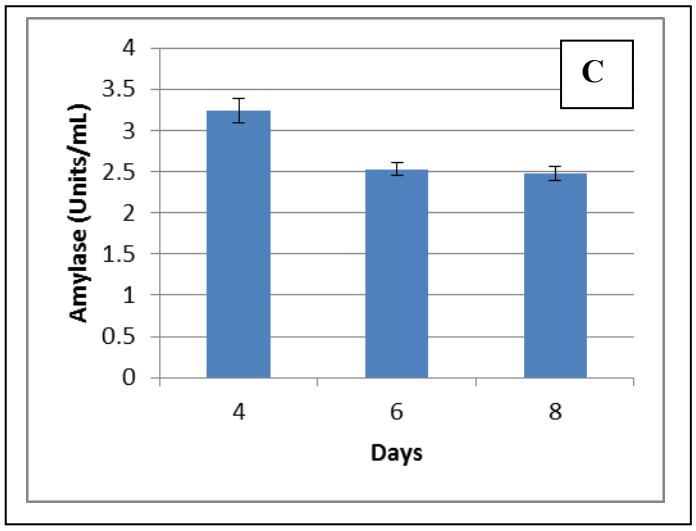


Fig. 1A-C: Cellulase, Xylanase and Amylase activities recorded in the first run of the bioreactor.

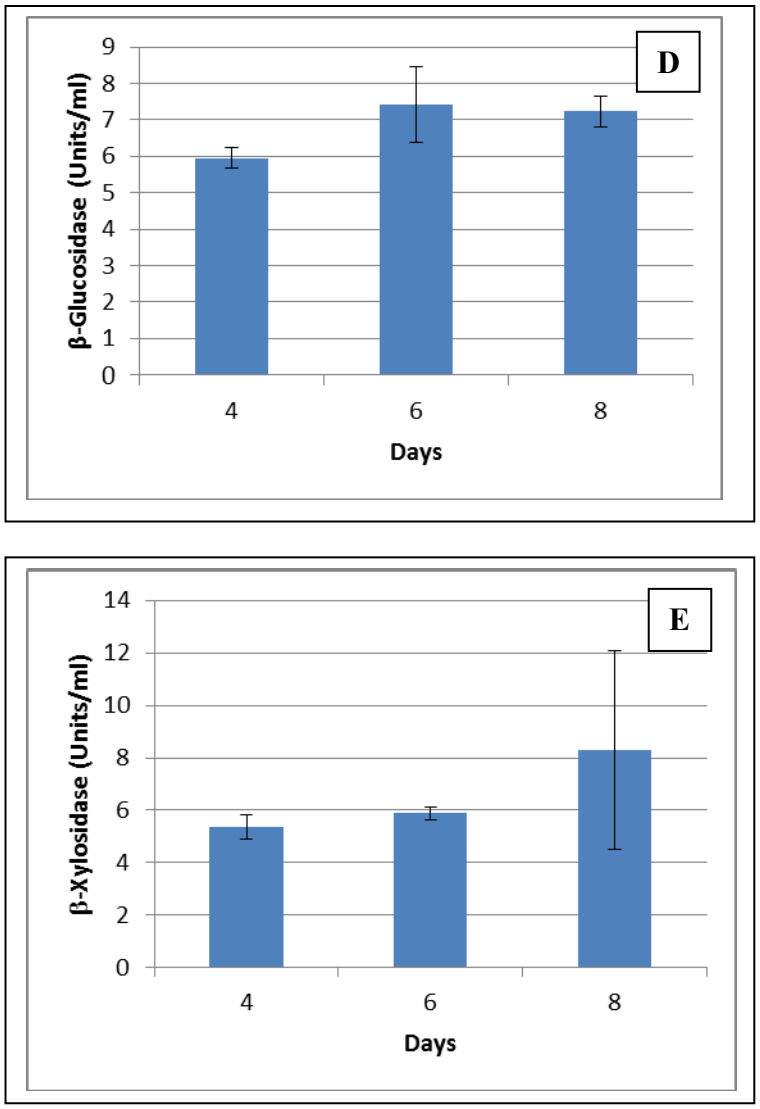


Fig. 1D and E: β -Glucosidase and β-Xylosidase activities recorded in the first run of the bioreactor.

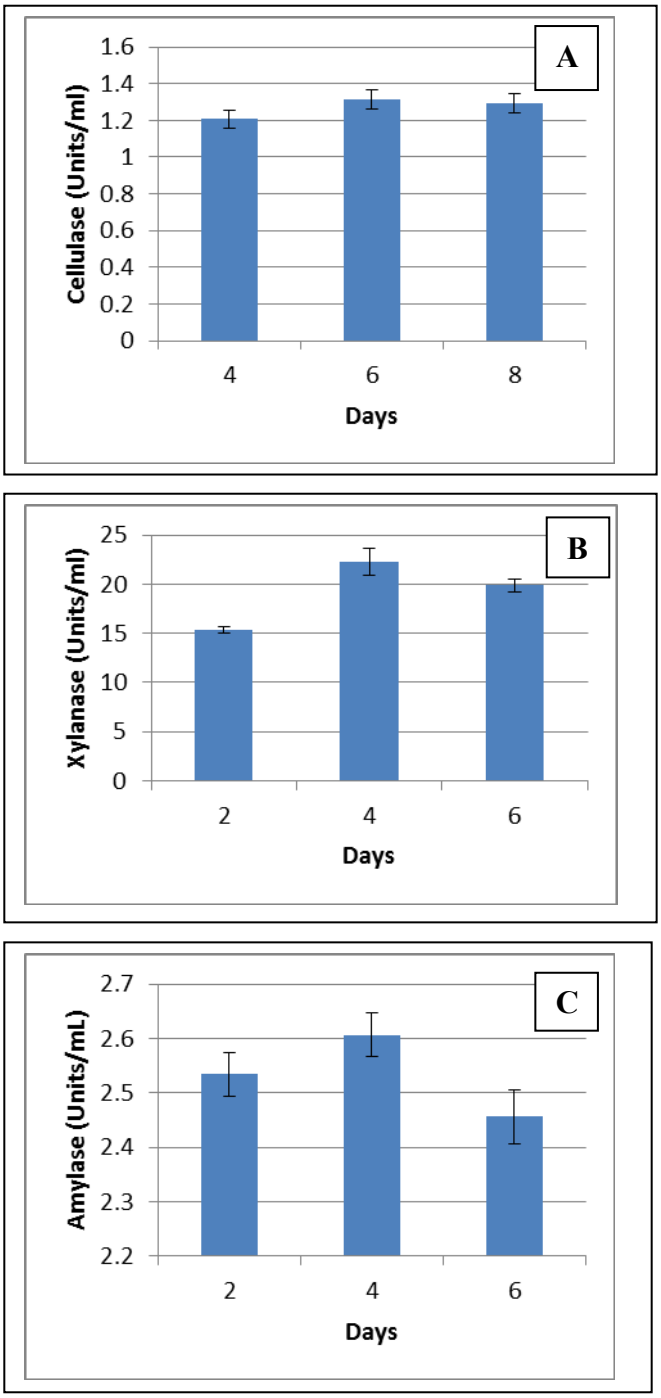


Fig. 2A-C: Cellulase, xylanase and amylase activities recorded in the second run of the bioreactor.

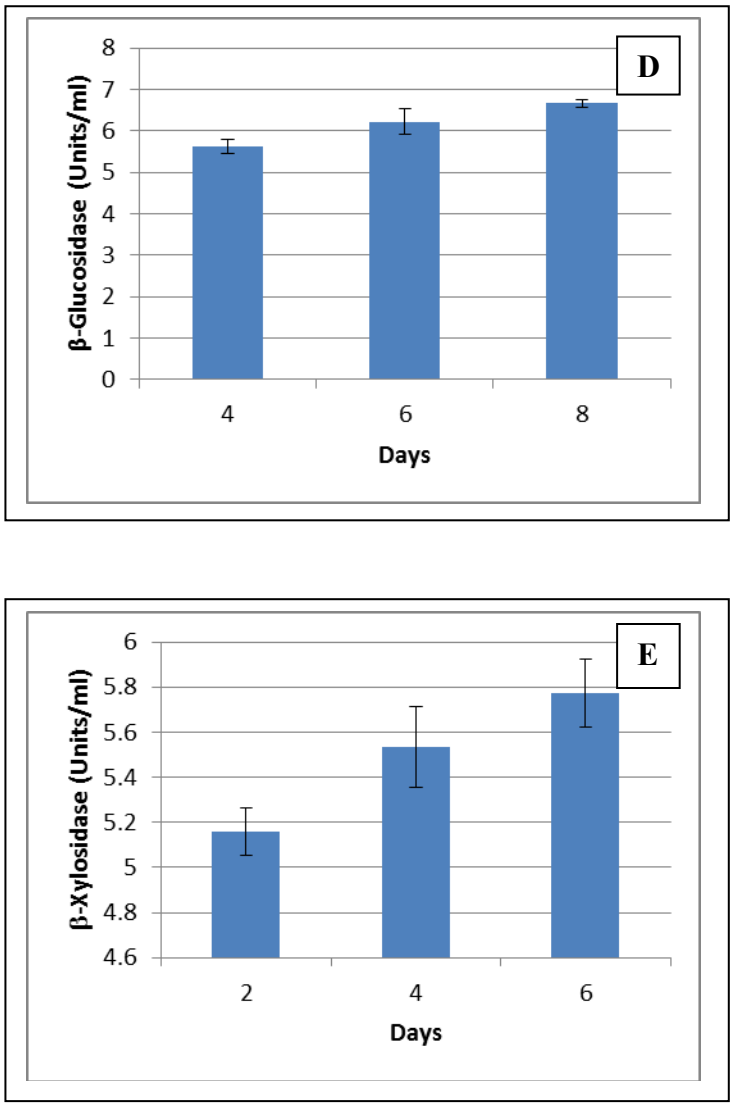


Fig. 2D and E: β-Glucosidase and β-Xylosidase activities recorded in the second run of the bioreactor.

Presentations

1. **Okeke, Benedict** and Nanjundaswamy, Ananda (2015). Research on development of farm/field-deployable bioreactor process for conversion of biomass to saccharides and ethanol. *Recent Advances in Fermentation Technology* (RAFT 11), Clearwater, FL November 8- 11. Presented November 9.

Conclusions

A bioreactor system for simultaneous saccharification and fermentation; and separate saccharification and fermentation of biomass to ethanol was developed for scale up and optimization of laboratory results. Three independent 200L scale up runs of the bioreactor

system for enzyme production did not yield the promising results achieved in the flask experiments. Thus further studies are required to optimize and scale up the process. Interestingly, results from flask experiments showed that use of starch-rich waste materials and other carbohydrate wastes in combination with pretreated biomass are required to make scale up of the process economically attractive. A patent, US 9,617,574 B2, was issued for aspects of the project.

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1. Focht, D.D. (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, WI, pp 407–426.
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