Production of Designer Xylose-Acetic Acid Enriched Hydrolysate from Bioenergy Sorghum, Oilcane, and Energycane Bagasses

Ming-Hsun Cheng[1,2], Shuchi Singh[1], Aidan N. Carr Clennon[1], Bruce S. Dien[3], Vijay Singh[1*]

[1]Department of Agricultural and Biological Engineering, University of Illinois Urbana-Champaign, Urbana IL 61801, USA

[2] Department of Natural Resources and Society, College of Natural Resources, University of Idaho, 995 MK Simpson Blvd, Idaho Falls, ID 83401, USA

[2]Center for Advanced Bioenergy and Bioproducts Innovation (CABBI), University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[3]United States Department of Agriculture (USDA), Agricultural Research Service (ARS), National Center for Agricultural Utilization Research (NCAUR), Bioenergy Research Unit, 1815 North University Street, Peoria, IL 61604, USA

Abstract

Xylan accounts for up to 40% of the structural carbohydrates in lignocellulosic feedstocks. Along with xylan, acetic acid in sources of hemicellulose can be recovered and marketed as a commodity chemical. Through vibrant bioprocessing innovations, converting xylose and acetic acid into high-value bioproducts via microbial cultures improves the feasibility of lignocellulosic biorefineries. Enzymatic hydrolysis using xylanase supplemented with acetylxylan esterase (AXE) was applied to prepare xylose-acetic acid-enriched hydrolysates from bioenergy sorghum, oilcane, or energycane using sequential hydrothermal-mechanical pretreatment. Various biomass solids contents (15 to 25%, w/v) and xylanase loadings (140 to 280 FXU/g biomass) were tested to maximize xylose and acetic acid titers. The xylose and acetic acid yields were significantly improved by supplementing with AXE. The optimal yields of xylose and acetic acid were 92.29% and 62.26% obtained from hydrolyzing energycane and oilcane at 25% and 15% w/v biomass solids using 280 FXU xylanase/g biomass and AXE, respectively.

Keywords: Xylose, Acetic acid, Xylanase, Acetylxylan esterase, Sequential hydrothermal-mechanical pretreatment

1. Introduction

Hemicellulose is the second most abundant structural carbohydrate after cellulose present in plants. In woody dicots and monocots, hemicellulose is composed of a xylan backbone (e.g., 1,4 linked β-D-xylopyranosyl linkages) substituted with acetyl, feruloyl, glucopyranosyl, *p*-coumaroyl, 4-*O*-methyl-D-glucuronopyranosyl, or α-arabinofuraosyl side chain groups with varying degrees (Bhardwaj et al., 2019). Applications of hemicellulose are highly desired in the context of lignocellulosic biorefining industries; however, hemicelluloses are underutilized in the industry due to their heterogeneous composition (Rojas-Pérez et al., 2022). Therefore, an intelligent design of biomass valorization is required to realize the full value of this abundant source of carbohydrates.

Xylan is the main component of hemicellulose and can be used to produce high-value bioproducts in lignocellulosic biorefinery. Xylooligosaccharide (XOS) and xylose monomer produced from xylan hydrolysis are used by the food, pharmaceutical, chemical, and biofuel industries (Brienzo et al., 2009; Forsan et al., 2022). The xylose fermentations have been widely investigated to produce organic acids, alcohols, lipids, biohydrogen, surfactants, furans, etc. (Gómez Millán et al., 2019; Kim et al., 2022; Narisetty et al., 2021; Oreoluwa Jokodola et al., 2022; Panjiar et al., 2020; Yu et al., 2022). Acetic acid traditionally has been a concern within the fermentation industry because it inhibits the growth of yeast. However, *S. cerevisiae* has now been metabolically engineered to co-utilize xylose and acetic acid at high concentrations to increase yields of biofuel, carotenoids, and vitamin A (Cheng et al., 2020a; Sun et al., 2019; Wei et al., 2015). These advances create an opportunity for advanced bioprocessing design to tailor the production of cellulosic syrups composed of specific sugars and organic acids for engineered microorganisms.

An efficient pretreatment process is required to disrupt biomass structure to facilitate the recovery of monosaccharides through enzymatic hydrolysis. Pretreatments are classified as physical, chemical, or biological, all of which have been thoroughly reviewed in the literature (Chen et al., 2022; Jędrzejczyk et al., 2019; Sharma et al., 2019). Green hydrothermal technology has gained interest for industrial applications because it is cost-effective, has low environmental impacts, and requires simple downstream and waste-water processing (Chen et al., 2022). Combining pretreatment unit operations further facilitates biomass deconstruction. In this case, a sequential hydrothermal-mechanical milling pretreatment has been developed that allows for high cellulosic sugar recoveries while minimizing the formation of inhibitors (e.g., furans) (Cheng et al., 2019; Mankar et al., 2021). Moreover, xylan is partially solubilized during the hydrothermal pretreatment and is typically converted to xylose via acid hydrolysis (Cheng et al., 2020a). Additionally, alkaline processes are also used to extract xylan (Sporck et al., 2017). Meanwhile, the acetyl groups can be recovered from the alkaline prehydrolysis liquors to improve hexose yields and eliminate potent fermentation inhibitors (Abdulrahman et al., 2019; Galeotti et al., 2018). Xylose can be also recovered using enzymes instead of acids, which has the advantage of eliminating furfural production associated with chemical hydrolysis (Sporck et al., 2017). Furthermore, enzymatic deacetylation using acetylxylan esterase (AXE) allows for the recovery of acetic acid from biomass (Razeq et al., 2018). To date, applying AXEs in cellulosic syrup preparation is rarely reported because few of them have been purified and characterized (Bajpai, 2022). Deacetylation and biomass saccharification are typically conducted in separate operations. Hence, the simultaneous production of xylose-acetic acid-enriched syrup from lignocellulosic feedstock by single step enzymatic hydrolysis is yet to be investigated.

Bioenergy sorghum (BSG), oilcane (OC), and energycane (EC) have been developed and utilized as energy crops for biofuel and platform chemical productions due to their high biomass yields (Cheng et al., 2021; Maitra et al., 2022; Maitra and Singh, 2021). These feedstocks consist of 30-39% glucan, 15-22% xylan, and 2-5% acetyl groups, and all have been studied to produce biofuels and platform chemicals (Cheng et al., 2020b; Cheng et al., 2019). However, low product titers and high costs are the major hurdles to overcome for biorefinery commercialization. An efficient bio-catalysis process combined with a hydrothermal pretreatment process has the potential to increase product productivity and lower production costs. For this reason, more research is warranted to develop technologies that produce designer hydrolysates paired with specific engineered microorganisms for the production of high-value bioproducts.

In this study, BSG, OC, and EC were used as model sources of biomass for the biocatalysis process design to produce xylose-acetic acid enriched hydrolysate, which can be readily used for engineered *S. cerevisiae* cultures to produce high-value platform chemicals. The enzymatic hydrolysis was optimized to maximize the recoveries of xylose and acetic acid from the biomass. Additionally, high solids processing was applied to increase xylose and acetic acid concentration in order to achieve higher fermentation product titers.

2. Materials and Methods

2.1 Bioenergy Sorghum (BSG), Oilcane (OC), and Energycane (EC)

Bioenergy sorghum TAM17800 (BSG) and oilcane 1566 (OC) were cultivated in experimental field plots located at the University of Illinois energy farm (Urbana IL), and harvested in September 2018 and September 2021, respectively. The wild type energycane (EC) was kindly provided by the Center for Advanced Bioenergy and Bioproduct Innovation (CABBI, Urbana IL). The biomass was shredded during harvest. The shredded biomass was ambiently air-

dried to less than 10% (w/w) moisture. The dried biomass was then ground to the particle size of 3 mm using an industrial hammer mill (W-8-H Schutte-Buffalo Hammermill, Buffalo, NY). The ground biomass was stored in sealed containers at 4 °C.

2.2 Sequential hydrothermal-mechanical refining pretreatment (HMR)

A sequential chemical-free hydrothermal pretreatment followed by disc milling was applied in this study. The chemical-free hydrothermal pretreatment was performed using a pilot scale continuous reactor (SüPR•2G Reactors, AdvanceBio System LLC., Milford, OH). The moisture content of the biomass was adjusted to 50% w/w (db). The BSG, OC, and EC were treated at 190 °C for 10 min as recommended by previous studies demonstrating excellent sugar recoveries (~85%) with negligible inhibitors formation (Cheng et al., 2019; Maitra & Singh, 2021). During the pretreatment, the superheated stream was injected into the reactor directly, and the reaction temperature was controlled by the pressure of the saturated steam. As the reactor is a continuous flow, the reaction time was set by adjusting the biomass feeding rate of the auger in the reactor. After the reaction, the pretreated biomass was dried at 40°C until the moisture was 20% in a tray dryer (The National Drying Machinery Co., Philadelphia, PA).

A pilot scale burr mill (Meadows Mills, Inc., N. Wilkesboro, NC) was used to disc mill the dried pretreated biomass. The burr mill consists of a stationary disc and a rotating disc (6 inches in diameter). The clearance between the discs was set to the minimum allowable gap, and the biomass was passed through the mill once (Cheng et al., 2020b).

2.3 Biomass composition analysis

The composition analysis of raw and pretreated biomass was performed to investigate chemical composition change before and after the sequential pretreatment. To prevent further

chemical composition changes in the hydrothermally pretreated biomass during the drying process, the biomass was first freeze-dried using a lyophilizer (Labcono, Kansas City, MO) for 72 h. The extractives, structural carbohydrates, lignin, and ash contents were determined following NREL protocols (Sluiter et al., 2008a, 2008b, 2008c).

2.4 Enzymatic hydrolysis

Xylose and acetic acid recoveries from lignocellulosic biomass were optimized in this study. The effect of varying solids loading at 15%, 20%, and 25% w/v was investigated. Fed-batch enzymatic hydrolysis was applied to improve mixing, which was critical when solids exceeded 20%. The enzymatic hydrolysis was performed in 125 ml Erlenmeyer flasks with a total reaction volume of 50 ml. The reactions were conducted for 96 h in an incubator shaker set at 60°C and 180 rpm.

The biomass loading pattern used in this study was adjusted according to our previous study to produce concentrated cellulosic syrups (Cheng et al., 2020b). The initial biomass loading was set at 15% w/v, and the remaining biomass was added at six-hour intervals (Table 1). For the enzymatic hydrolysis, 1 M (pH 5.0) sodium citrate buffer was added to a final concentration of 50 mM. Xylanase (NS22244, Novozymes North America, Inc., Franklinton, NC, USA) and experimental acetylxylan esterase (3.1.1.72) (kindly provided by International Flavors & Fragrances Inc., Cedar Rapids, IA, USA) were used. Three xylanase dosages (140, 210, 280 fungal xylanase unit (FXU)/g biomass) were compared, and the AXE loading was added at 25% of xylanase (Table 2).

Additionally, the surfactant, PEG 4000 (Sigma-Aldrich), was added at 2% (w/w) of the total biomass to secure proper mass transport (Cheng et al., 2020b). The sodium citrate buffer,

enzymes, and surfactant were added at the beginning of the reactions. DI water was added to bring the slurry to the desired solids contents.

2.5 Sugar and acetic acid analysis

Approximate 0.5 ml of sample was collected from the enzymatic hydrolysis reaction and centrifuged at 9000×g for 10 min. The supernatants were filtered through a 0.2 μm PTFE filter prior to HPLC analysis. Sugars, organic acids, and furan concentrations were determined using an HPLC system (Bio-Rad Aminex HPX-87H, Biorad, Hercules, CA). The xylose and acetic acid yields were calculated based on the raw biomass chemical composition following Eq. 1, 2 (Sluiter et al., 2008b).

$$Xylose\ yield\ (\%) = \frac{Xylose\ recovered\ (g)}{Xylan\ content\ (\%) \times total\ biomass\ (g) \times 1.13} \times 100\% \quad Eq.1$$

$$Acetic\ acid\ yield\ (\%) = \frac{Acetic\ acid\ recovered\ (g)}{Acetate\ content\ (\%) \times total\ biomass\ (g) \times 1.02} \times 100\% \quad Eq.2$$

2.6 Statistical analysis

Enzymatic hydrolysis experiments under different reaction conditions were performed in triplicate to yield xylose and acetic acid. The data were expressed as mean ± standard deviation of three values. The means were compared using analysis of variance (ANOVA) and Tukey tests based on a p-value of 5% ($p < 0.05$). The interactive effect of biomass solids loading and enzyme dosage on the xylose-acetic acid enriched hydrolysate preparation is investigated. Additionally, the response surface methodology (Table 3) with the central composite design was used to determine optimal reaction conditions for the designer hydrolysate preparation. All statistical analyses were performed using R (V.4.2.2).

3. Results and Discussion

3.1 Effect of HMR pretreatment on biomass composition alteration

The compositions (%, w/w, db) of raw and HMR pretreated biomass samples are listed in Table 4. BSG, OC, and EC have 14 - 22% xylan, 4 - 5% acetate, 29 - 39% glucan contents, and 16 - 22% lignin. After the HMR pretreatment, xylan, lignin, and acetate contents were reduced to 5 -11%, 8 - 14%, and 3 - 4%, respectively. During the hydrothermal reaction, hydronium ions are formed under high temperatures and pressure, allowing for acid catalytic hydrolysis of hemicellulose to produce soluble xylooligosaccharide and xylose (Jönsson and Martín, 2016). HMR pretreatment partially solubilizes xylan, alters lignin structure, and extracts acid soluble lignin (Cheng et al., 2019). In contrast, most of the glucan remained with the solids.

Additionally, this HMR pretreatment limited the inhibitor formations. The highest concentrations of formic acid, lactic acid, furfural, and 5-hydroxymethylfurfural are 0.23%, 0.06%, 0.04%, and 0.02%, respectively. They are all below the minimum inhibitor concentration of 0.4% to lower the efficiency of microbial cultures using engineered *S. cerevisiae*. These results are consistent with the previous study, and their inhibitory effects on the microbial cultures are negligible (Cheng et al., 2019). Therefore, the HMR pretreatment provides the needed flexibility to tailor separate sugar streams within the context of biorefineries.

3.2 Xylose yield

Figure 1 displays xylose recoveries (% of max) for pretreated BSG, OC, and EC at various enzyme and solids loadings, which is further discussed below.

3.2.1 Bioenergy sorghum (BSG)

The xylose yields from BSG were from 42% to 67% (of max). Supplementing the xylanase with AXE increased xylose yields by 2% to 13%. AXE supplementation has the greatest effect on xylose yield with a xylanase loading of 280 FXU/g biomass (Figure 1a).

Enzyme loading rates had a significant positive effect on xylose yields ($p = 5.73\times10^{-10}$). Adding AXE also significantly improved xylose yields ($p = 1.03\times10^{-7}$); the improvement was inversely correlated with solids loading. The xylose yield was improved by up to 13% at the highest enzyme loading at 280 FXU/g biomass with AXE. Xylanse and AXE loadings were positively synergistic for xylose yield ($p = 0.01$).

Additionally, solids loading is another critical parameter for hemicellulose hydrolysis ($p = 0.01$). Xylose yields declined as solids loadings were increased, probably due to poor mass transfer at higher solids content over 20% (w/v) (Cheng et al., 2020b). Xylose yields were reduced by 2 to 6% when the solids content was increased from 15% to 20%, and a further 2 to 4% decrease in xylose yield when biomass solids were increased from 20 to 25%. However, including AXE improved the xylose yields at the higher solids loadings over 20%. The maximum xylose yield of 66.5% was obtained with 20% biomass solids content digested with 280 FXU/g biomass of xylanase supplemented with AXE.

3.2.2 Oilcane (OC)

Better xylose yields were obtained from pretreated OC than BSG. The xylose yields were 56 to 70% with only xylanase, and adding AXE improved xylose yields by 10 to 22% (Figure 1b).

Xylose yields decreased with increasing biomass solids contents ($p = 2.36\times10^{-5}$). Xylose yields fell 4 to 5% when the solids content was increased to 20%, and another 1%

decrease at 25% solids. Moreover, increasing biomass solids content resulted in a small range of xylose yield improvements from adding AXE. Similar xylose yields (79 to 83%) were observed at 20% and 25% solids contents with enzyme loadings of 210 and 280 FXU/g biomass when supplemented with AXE. The maximum xylose titer of 55.45 g/L was obtained at 25% biomass solids loading for enzymatic hydrolysis.

Increased enzyme dosage ($p = 7.18\times10^{-13}$) and AXE supplementation ($p < 2.20\times10^{-16}$) played important roles in improving xylose yields. Xylose yields increased by 8%, 12%, and 8% when the xylanase loading was increased from 140 to 280 FXU/g for the reactions with biomass solids content at 15%, 20%, and 25%, respectively. Furthermore, AXE raised the xylose yield by 6 to 8% with increasing enzyme loadings. The maximum OC xylose yield of 88.10% was obtained using 15% biomass solids at 280 FXU/g biomass of xylanase supplemented with AXE.

3.2.3 Energycane (EC)

Pretreated EC had similar trends as OC (Figure 1c). The solids loading was a critical factor affecting xylose yields ($p = 1.11\times10^{-10}$). The xylose yields decreased by 10 to 12% when solids loadings were increased from 15% to 20%. Another 3 to 7% reduction in xylose yields was observed when the solids loading was increased to 25%. The xylose yield decreased from 82 % to 61% when the biomass solids content increased from 15% to 25%.

Including AXE improved xylose yields ($p < 2.2\times10^{-16}$) by 12 to 31% and was positively correlated with biomass solids. The maximum xylose yield of 92.3% was obtained at 25% solids content. At 15% solids, the xylose yields were slightly increased (8%) by including AXE, reaching about 80 to 83%, which were close to the results observed from the

reactions with higher biomass solids contents (25%). These results demonstrate that the xylose-enriched hydrolysates can be prepared using xylanase supplemented with AXE at high biomass solids loadings (~25%), and provide xylose concentrations greater than 49 g/L.

EC provided the highest xylose yields followed by OC and BSG. These differences found among these samples are not unexpected given the complexity of the plant cell wall and can be probably attributed to differences in active surface area, crystallinity, and porosity (Cheng et al., 2022). Moreover, the degree of covalent bonding between lignin and hemicellulose is expected to be another critical parameter for xylose recovery from the feedstocks (Brienzo et al., 2009).

3.3 Acetic acid yield

Acetic acid is an important co-product of xylose extraction that is released from the cleavage of acetyl groups linked to xylan. Acetate yields are shown in Figure 2 and discussed below.

3.3.1 Bioenergy sorghum (BSG)

Acetic acid was co-related with xylose at yields of 13 to 29% (Figure 2a). Solids loading had a significant effect on acetic acid yields ($p = 6.83\times10^{-12}$). Mass transfer and proper mixing are critical for xylose-acetic acid enriched hydrolysate preparation due to the slow liquefaction of the biomass slurry by xylanase. Acetic acid yields decreased by 6 to 8% when solids content increased from 15% to 25% without the addition of AXE.

Applying a higher xylanase dosage (280 FXU/g biomass) improved acetic acid yields by 5 to 12% ($p = 2.89\times10^{-12}$). It increased the acetic acid yield from 19.5% to 22.1% for the reaction with 15% solids; however, the yields decreased to about 15% for the hydrolysis with

25% solids content. AXE is expected to directly improve acetic acid yields by saponifying the ester linkages of the acetyl group to xylan. AXE significantly improved the acetic acid yields by up to 12% compared to solely adding xylanase ($p < 2.2\times10^{-16}$). In the reactions with 15% solids, the acetic acid yield was improved by adding AXE, especially at the higher enzyme loading (280 FXU/g biomass). For the higher solids loading hydrolyses (20% and 25%), acetate yields were not significantly improved by increasing the enzyme loading from 210 FXU to 280 FXU/g biomass. These results suggest with the reactions were mass transferred limited at higher biomass solids. The maximum acetic yield of 29.23% was obtained at 15% solids using 280 FXU xylanase/g biomass supplemented with AXE.

3.3.2 Oilcane (OC)

Higher acetic acid yields of 26 to 60% were observed from OC hydrolysis (Figure 2b). Acetate yield was negatively affected by increased solids loadings ($p = 1.79\times10^{-7}$). However, the solids loading did not affect acetic acid yield when xylanase was only added. The acetic yields were from 26 to 40%, and a 3 to 5% yield reduction was found at 25% solids with higher enzyme loadings (210 FXU and 280 FXU/g biomass).

Adding AXE improved acetic acid yields ($p < 2.2\times10^{-16}$). The yield doubled for reactions run with 140 FXU xylanase/g biomass. There were 21 to 28% and 19 to 22% increases observed for reactions using 210 FXU and 280 FXU/g biomass, respectively. The maximum yield of 62.3% was achieved by hydrolyzing 15% solids with 280 FXU xylanase/g biomass supplemented with AXE. Compared to SBG, the higher acetic acid yields could be related to more xylan (68%) and acetyl group (35%) being solubilized during HMR pretreatment (Table 4).

Additionally, the effect of the HMR pretreatment can possibly explain the similar acetic yields obtained when hydrolyzing OC at various solids contents without adding AXE. The effectiveness of HMR in breaking apart plant fibers should partially mitigate the negative impact of increased solids contents on the release of acetyl groups. Moreover, increased biomass surface area (~25%) afforded by HMR would make the acetyl groups accessible to the esterase (Cheng et al., 2022). This is particularly important because the reaction was conducted without cellulases. Therefore, higher acetic acid yields are correlated with compositional changes following HMR pretreatment.

3.3.3 Energycane (EC)

EC hydrolysis yielded acetic acid of 22 to 56% (Figure 2c). Higher solids loading resulted in decreasing acetic acid recovery ($p = 5.42\times10^{-14}$). The best yield was found in the hydrolysis with 15% biomass solids content. An approximate 10% decrease in acetic acid yield was observed when the solids content increased from 15% to 25%.

Acetic acid yields were positively correlated with enzyme loading ($p = 2.03\times10^{-6}$), especially for 15% biomass solids hydrolyses. AXE improved acetic yields by 15 to 25% ($p< 2.2\times10^{-16}$). The yields doubled for the reactions hydrolyzing 20% and 25% biomass solids content using 210 FXU and 280 FXU/g biomass. The maximum yields were higher than 52%; again similar to that observed for the EC hydrolysis. HMR pretreatment was highly effective at releasing xylan (71%) and acetyl groups (47%) (Table 4).

3.4 Glucose yield

Glucan is the major structural carbohydrate within the plant cell wall, which is entwined around hemicellulose and lignin. Therefore, the release of glucose during biomass

hydrolysis for producing xylose-acetic acid-enriched syrups is unavoidable. The effect of xylanase and AXE on glucose yield was also investigated in this study. Figure 3 illustrates glucose yields for BSG, OC, and EC hydrolysates.

3.4.1 Bioenergy sorghum (BSG)

Glucose production was limited because cellulase was not used during hydrolysis. According to the results (Figure 3a), BSG glucose yields were from 9 to 33%. As observed for xylose and acetic acid recoveries, higher solids resulted in lower glucose yields ($p = 8.90\times10^{-11}$). The glucose yields decreased by 5 to 10% when the solids contents were increased from 15% to 20% and 25%. Similar glucose yields were observed at 20% and 25% solids without adding AXE.

Increasing xylanase loading improved the glucose yield ($p< 2.2\times10^{-16}$), especially for the reactions at 15% solids content. A 10% increase in glucose yield was obtained when the xylanase loading increased from 140 FXU to 280 FXU/g biomass for the hydrolysis of 15% biomass solids content. The addition of AXE significantly increased glucose yields ($p < 2.2\times10^{-16}$). There was a 10 to 16% improvement in the glucose yield at higher solids contents (25%), and the maximum yield was over 32%.

Though xylanase is expected to target xylan glycosidic bonds, it is possible that the crude – commercial grade enzyme used in this study had limited cellulase activity. However, glucose release was limited and slow biomass liquefaction resulted in lower BSG glucose yields than OC and EC.

3.4.2 Oilcane (OC)

The glucose yields of OC hydrolyses were affected by biomass solids loading ($p < 2.2\times10^{-16}$). Glucose yields decreased by 2% to 8% at high solids loadings (25%). The yields increased with higher xylanase loadings, especially for the reactions at 20% and 25% solids contents. A glucose yield greater than 30% was observed at 25% solids hydrolysis using 280 FXU/g biomass (Figure 3b).

AXE improved glucose yields ($p < 2.2\times10^{-16}$). For the reactions with 15% and 20% solids contents, glucose yields increased by at least two folds with AXE supplementation. The maximum yield of 69.1% was obtained at 15% solids hydrolysis and the highest enzyme loading. Though the glucose yields reduced with increasing biomass solids content, AXE facilitated glucose release for the reactions with higher solids hydrolyses. Based on the results, the yield derived from 20% solids hydrolysis with high enzyme loading was similar to the 15% solids hydrolysis with 210 FXU xylanase/g biomass and AXE. These results concur with earlier results showing biomass deacetylation facilitates glucose recovery from cellulose (Wang et al., 2020). Additionally, partial glucan solubilization caused by HMR pretreatment would promote glucose release during the hemicellulose hydrolyses using xylanase and AXE.

3.4.3 Energycane (EC)

Similar trends to OC glucose yields were found in EC hydrolysates (Figure 3c). Biomass solids content ($p < 2.2\times10^{-16}$) and xylanase loading ($p = 1.02\times10^{-16}$) are critical factors that affect glucose yields. Low biomass solids and high xylanase loading led to a better glucose yield. The yields were 6 to 30% from the reactions with xylanase, which are similar to BSG and slightly lower than OC. There was a 7 to 20% decrease in glucose yields when the solids content increased to 25%, and about a 3 to 10% increase was observed when the xylanase loading increased to 280 FXU/g biomass.

Glucose yields for EC were doubled by adding AXE, and the maximum yield of 61.4% was obtained at 15% solids loading with 280 FXU xylose/g biomass supplemented with AXE. Similar to that observed for other biomasses, glucose yields decreased when biomass solids contents were increased. The glucose yield at 20% solids hydrolysis with 280 FXU xylanase/g biomass supplemented with AXE was 47.0%, which was similar to the yield observed at 15% solids hydrolysis with 210 FXU xylanase/g biomass supplemented AXE. This result indicates that AXE mitigates the effects of high biomass loading. Due to the simultaneous recoveries of xylose, acetic acid, and glucose during the hemicellulose hydrolysis, choosing proper biomass solids contents and enzyme loadings are crucial for controlling the composition of designer cellulosic syrups produced through enzymatic processing.

3.5 Discussion

3.5.1 Correlation between xylose and acetic yields

The correlation between sugars and acetic acid released during hemicellulose hydrolysis is critical for enzymatic hydrolysis to tailor the production of the desired products. Figure 4 depicts the correlation between xylose, glucose, and acetic acid yields.

For BSG and OC, there was a positive correlation ($r > 0.7$) bwteen xylose, glucose, and acetic acid yields. These results demonstrate that hemicellulose hydrolysis with xylanase and AXE simultaneously facilitates glucose release. Moreover, no inhibitory effects were observed between cellulosic sugars and acetic acid in hemicellulose hydrolyses. Additionally, a higher correlation coefficient was observed between acetic acid and glucose yields. This indicates that deacetylation could facilitate glucose release during hemicellulose hydrolyses, which is consistent with the results shown earlier (Figure 3).

However, a poor correlation between xylose and acetic acid yields (r = 0.35) was found for pretreated EC. A similar result was also observed between xylose and glucose yields (r = 0.39). Based on EC chemical composition changes after HMR pretreatment (Table 4), approximately 47% of acetate was removed by the HMR process, which is the highest reduction in acetate content among these feedstocks. Moreover, greater xylan solubilized by HMR pretreatment led to higher xylose release during hydrolysis. Due to the larger amounts of xylan and acetate solubilized during HMR pretreatment compared to OC and BSG, the correlation between xylose and acetic acid yields by adding acetylxylan esterase (AXE) was not seen in the results. Different results observed with EC are likely due to significant chemical compositional changes resulting from the HMR pretreatment and the variances in plant cell wall structures among the three biomass samples. Further investigations are needed to reveal the variation in biomass structures of transgenic energy crops.

3.5.2 Biomass Solids and enzyme loadings

Based on xylose acetic acid yields, biomass solids content, and enzyme loading played critical roles in determining the efficiency of hemicellulose enzymatic hydrolysis. The response surface experimental design was used to determine the optimal biomass solids and enzyme loadings for targeted xylose-acetic enriched designer cellulosic hydrolysates.

Optimal biomass solids loadings for hemicellulose hydrolyses are shown in Figure 5. For BSG, the best model ($p = 0.005$, $r^2 = 0.755$) for the prediction of xylose yield includes the variables of biomass solids content (X), enzyme loading (Y), and their pure quadratic (PQ) terms (Eq. 3). The best reaction condition is predicted to be 18.0% biomass solids content with 280 FXU xylanase/g biomass and AXE resulting in 64.4% of xylose yields. Solids content, enzyme loading, and the PQ of solids content (Eq.4) contributed to the OC xylose model

notably ($p = 7.56\times10^{-6}$, $r^2 = 0.902$). The best xylose yield of 82.9% is predicted from the reaction applying 24.2% biomass solids and 280 FXU xylanase/g biomass and AXE. For EC xylose yield prediction, biomass solids content, enzyme loading, and the interaction of biomass solids and enzyme loading contributed to the EC model significantly ($p = 5.72\times10^{-3}$, $r^2 = 0.74$). The optimal xylose yield of 82.7% is predicted from the reaction using 12.6% solids and 246.5 FXU xylanase/g biomass and AXE (Eq. 5).

$$Xylose\ Yield_{BSG} = 55.59 - 1.33X + 7.01Y - 1.65X^2 + 1.49Y^2 \qquad Eq.3$$

$$Xylose\ Yield_{OC} = 81.13 - 2.82X + 2.93Y + 1.69X^2 \qquad Eq.4$$

$$Xylose\ Yield_{EC} = 82.12 - 0.43X + 3.83Y + 2.58XY \qquad Eq.5$$

The results of acetic acid yield optimization are graphed in Figure 6. Biomass solids (X), enzyme loading (Y), and PQ (X^2) of biomass solids are the variables contributing to significant models to predict acetic acid yields from BSG, OC, and EC. Based on the BSG model (Eq. 6, $p = 2.51\times10^{-4}$, $r^2 = 0.81$), the maximum acetic acid yield of 26.0 is predicted from hydrolyzing 19.2% with 210 FXU xylanase/g biomass and AXE. For the OC acetic acid (Eq. 7, $p = 4.49\times10^{-4}$, $r^2 = 0.82$), the hydrolysis of 12.0 % biomass solids with 210 FXU xylanase/g biomass and AXE is predicted to have the best yield of 59.5%. A similar model was observed from EC (Eq. 8). From the EC model (Eq. 8, $p = 2.26\times10^{-4}$ $r^2 = 0.87$), the hydrolysis of lower biomass solids loading of 6.85% with 210 FXU xylanase/g biomass and AXE is predicted to have the best acetic yield of 58.5%.

$$Acetic\ Acid\ Yield_{BSG} = 26.11 - 0.43X + 2.96Y - 1.30X^2 \qquad Eq.6$$

$$Acetic\ Acid\ Yield_{OC} = 57.46 - 2.53X + 2.64Y - 0.79X^2 \qquad Eq.7$$

$$Acetic\ Acid\ Yield_{EC} = 53.72 - 3.60X + 2.05Y - 0.68X^2 \qquad Eq.8$$

4. Conclusion

Xylose-acetic acid enriched syrups were produced from BSG, OC, and EC by enzymatic hydrolysis using xylanase and AXE. The HMR pretreatment facilitated xylan and acetyl groups solubilization and released during hydrolysis. Xylose yields significantly increased with increased xylanase loadings. Adding AXE significantly improved both xylose and acetic acid yields. Notably, AXE supplementation doubled acetic acid yields for oilcane and energycane hydrolysates. HMR was most effective at releasing xylose and acetic acid for OC and EC. The maximum yields of xylose and acetic acid were 92.3% and 59.8% obtained from energycane and oilcane hydrolyses supplemented with AXE, respectively.

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Table 1 Biomass loading pattern of the fed-batch enzymatic hydrolysis

Target solids content w/v	Initial solids loading	6h	12h
15%	15%		
20%	15%	5%	
25%	15%	5%	5%

Table 2 Enzymes loading for the designer hydrolysate preparations

	Xylanase	Acetylxylan Esterase (AXE)
Low dosage	140 FXU/g biomass*	25% of 140 FXU xylanase loading
Medium dosage	210 FXU/g biomass	25% of 210 FXU xylanase loading
High dosage	280 FXU/g biomass	25% of 280 FXU xylanase loading

*The base scenario of xylanase loading is based on Zhao et al's (2020) study on ethanol production from high solids processing (Zhao et al., 2020).

Table 3 Coded and actual level of the two independent variables for the production of xylose-acetic acid enriched hydrolysates

		Code and actual level		
	Code	-1	0	1
Solids loading (%)	X	15	20	25
Xylanase loading (FXU/g biomass)	Y	140	210	280

Table 4 Compositions (% w/w) of raw and HMR pretreated bioenergy sorghum (BSG), oilcane (OC), and energycane (EC)

		Extractives	Glucan	Xylan	AIL	ASL	Acetate	Ash
BSG*	Untreated	11.82±0.83	38.77±0.38	21.76±0.09	14.27±0.23	1.88±0.06	4.51±0.17	2.72±0.10
	HMR	28.13±0.57	40.72±1.02	10.28±0.54	12.84±0.68	0.90±0.17	4.02±0.07	4.49±0.08
OC[#]	Untreated	27.30±0.02	29.92±0.01	14.69±0.03	21.33±0.04	0.55±0.33	5.10±0.26	1.35±0.02
	HMR	43.53±0.47	29.72±0.71	4.82±0.18	8.57±0.03	0.04±0.02	3.36±0.11	0.47±0.04
EC[†]	Untreated	14.38±0.90	32.72±0.34	18.29±0.19	16.85±0.97	1.88±0.06	5.07±0.14	0.33±0.04
	HMR	35.74±0.94	33.33±0.48	5.22±0.86	13.59±0.71	0.87±0.02	2.68±0.27	0.39±0.03

AIL: acid insoluble lignin: ASL: acid soluble lignin. *:Cheng et al., (2019); #: Cheng et al., (2022); †: Maitra and Singh (2021)

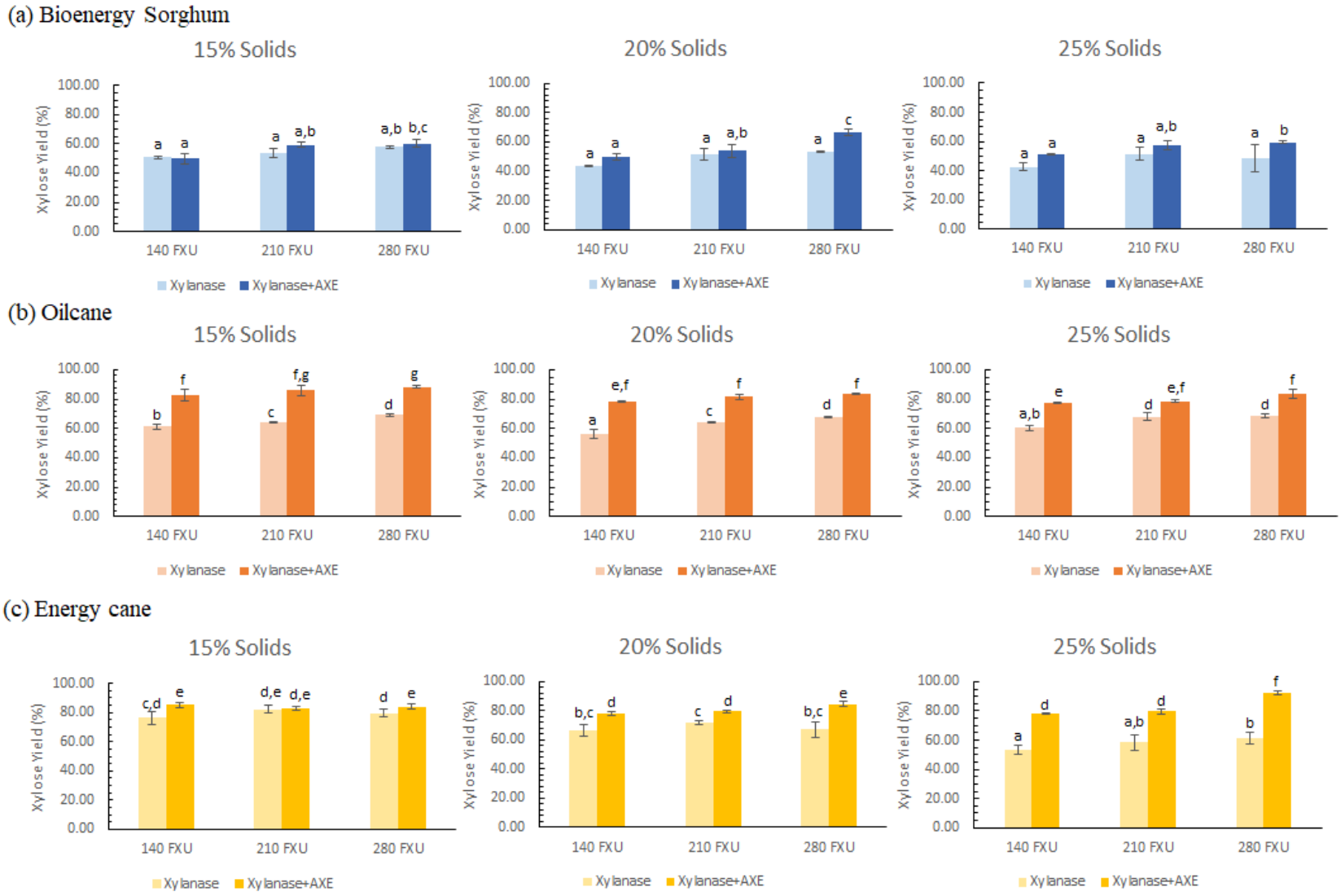

Figure 1 Xylose yields of pretreated (a) bioenergy sorghum, BSG; (b) oilcane, OC; and (c) energycane, EC, hydrolysates. Different letters on the bars represent significant statistical differences ($p < 0.05$) determined by Tukey's honest significant difference test.

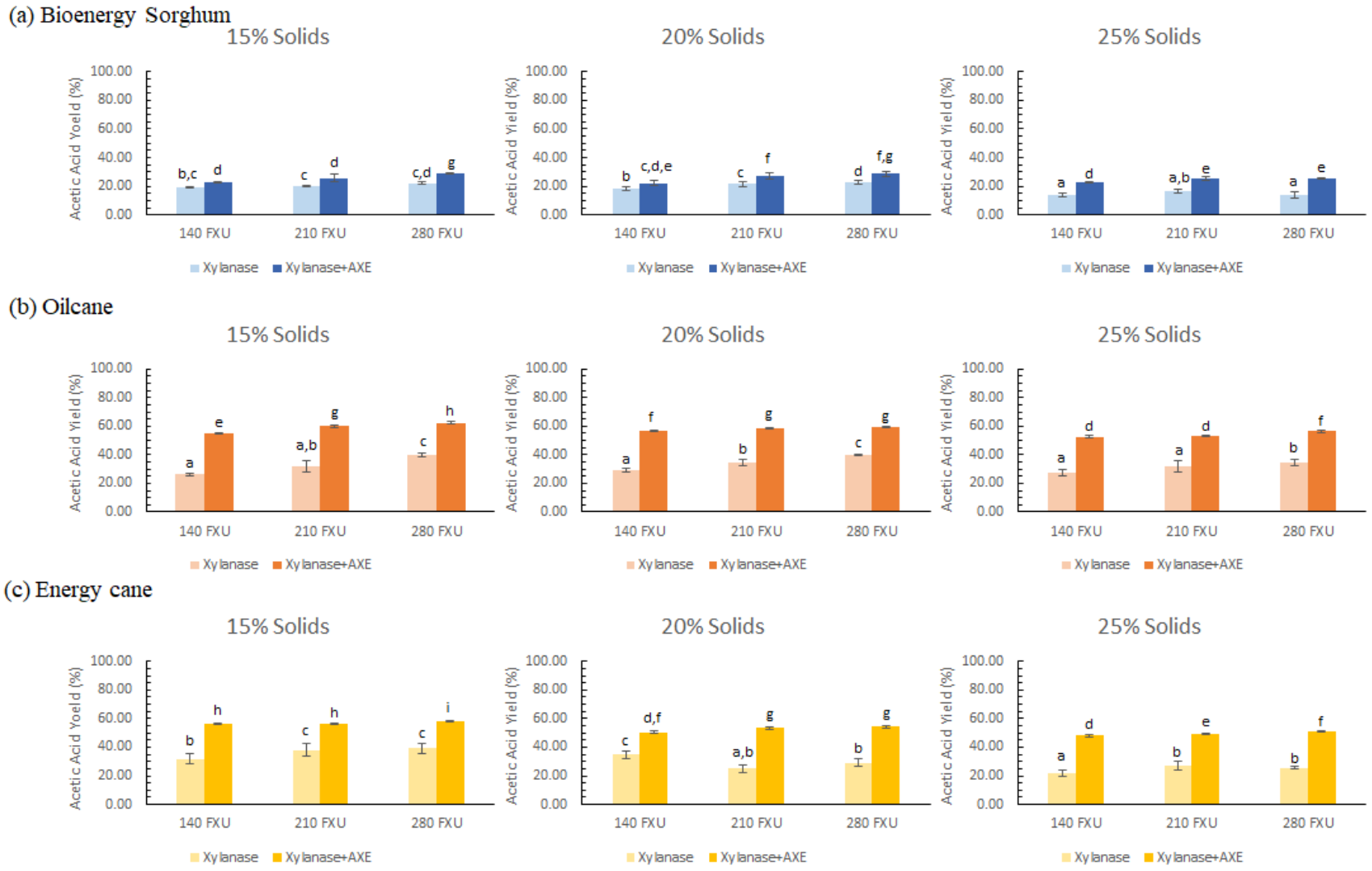

Figure 2 Acetic acid yields of pretreated (a) bioenergy sorghum, BSG; (b) oilcane, OC; and (c) energycane, EC, hydrolysates. Different letters on the bars represent significant statistical differences ($p < 0.05$) determined by Tukey's honest significant difference test.

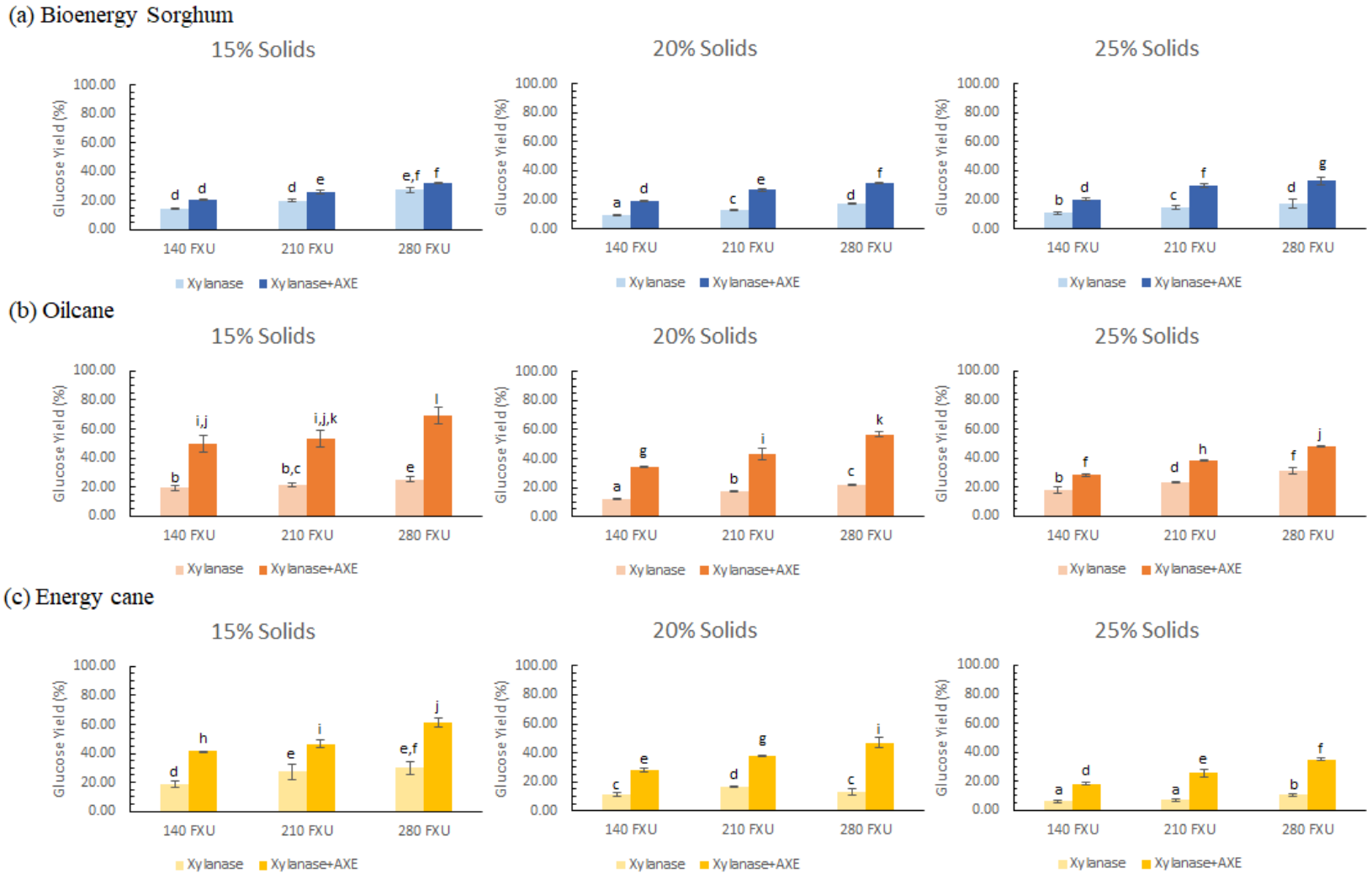

Figure 3 Glucose yields of HMR pretreated (a) bioenergy sorghum, BSG; (b) oilcane, OC; and (c) energycane, EC, hydrolysates. Different letters on the bars represent significant statistical differences ($p < 0.05$) determined by Tukey's honest significant difference test.

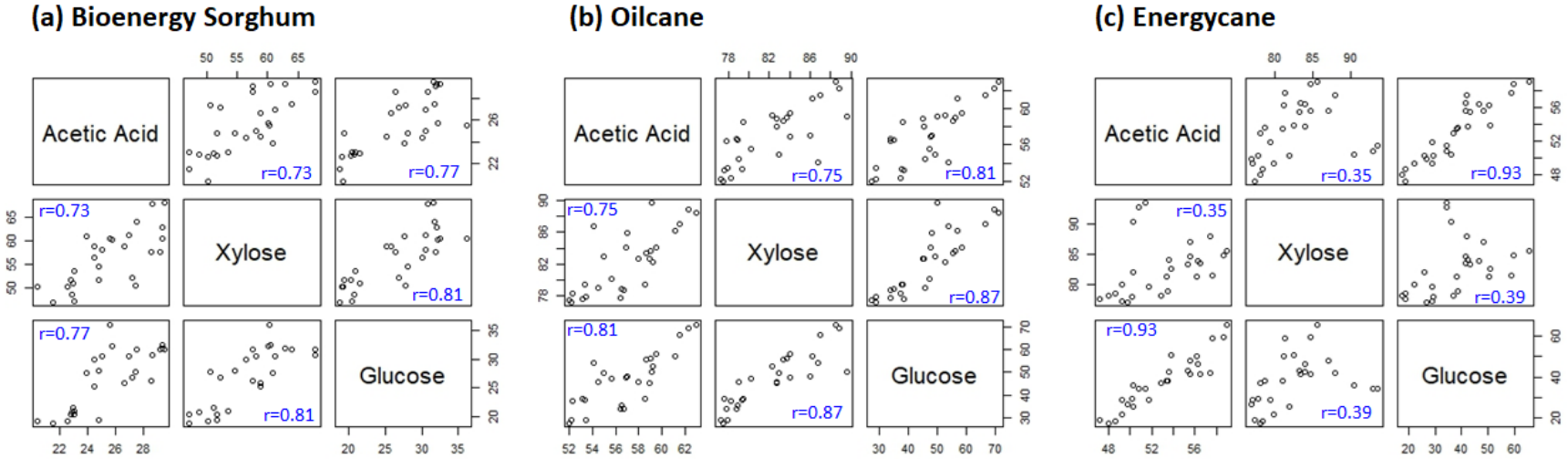

Figure 4 Correlation between xylose, glucose, and acetic yields: (a) Bioenergy sorghum; (b) Oilcane; and (c) Energycane.

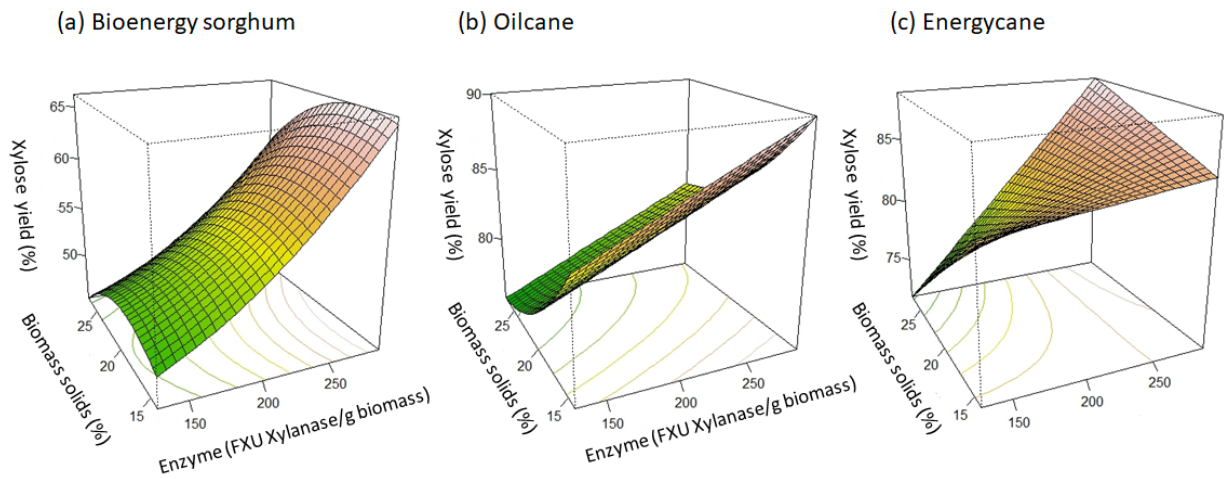

Figure 5 Contour plot of xylose yield of (a) bioenergy sorghum, BSG; (b) oilcane, OC; and (c) energycane, EC, hydrolyses.

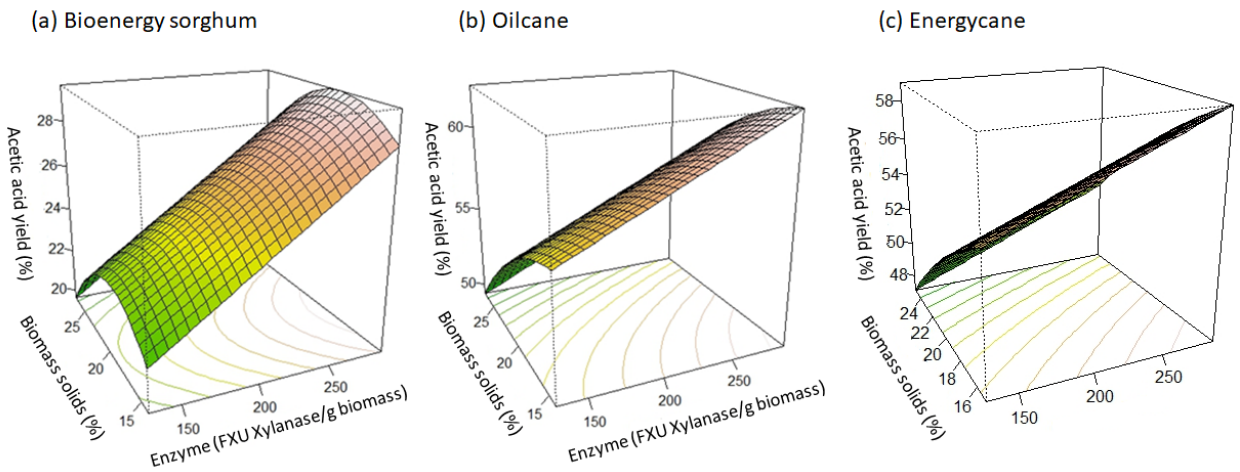

Figure 6 Contour plot of acetic acid yields of (a) bioenergy sorghum, BSG; (b) oilcane, OC; and (c) energycane, EC, hydrolyses.