Green Chemistry

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d5gc07076g

Optimization of pre-commercial enzyme dosage for a potential lignocellulosic biorefinery

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Received 31st December 2025,
Accepted 21st April 2026

DOI: 10.1039/d5gc07076g

rsc.li/greenchem

Lignocellulolytic enzymes remain one of the primary cost constraints in second-generation (2G) ethanol biorefineries. Achieving efficient hydrolysis of structural carbohydrates with minimal enzyme dosage, maintaining slurry fermentability for industrially relevant ethanol titers, and maximizing ethanol yield per ton of biomass are among the major challenges in 2G processes. In this study, we optimized the dosages of pre-commercial cellulase (NS22257) and hemicellulase (NS22244) on pilot-scale, hydrothermally pretreated lignocellulosic substrates. Enzyme dosages were evaluated at three levels: 20 mg of cellulase with 7.25 mg of hemicellulase (ED-1), 40 mg with 14.5 mg (ED-2), and 60 mg with 21.75 mg (ED-3). As expected, the highest sugar yields were obtained with ED-3; however, for sweet sorghum, oilcane, and miscanthus, sugar yields from ED-2 and ED-3 were not significantly different ($p < 0.05$). For example, sweet sorghum produced 123.78 ± 1.54 g L^{-1} and 125.76 ± 0.46 g L^{-1} of total sugars (glucose and xylose) with ED-2 and ED-3, respectively. Although energycane exhibited a statistically significant difference between ED-2 and ED-3, the incremental gain with ED-3 was modest, increasing sugar release by only 9.02 g L^{-1} relative to ED-2. Importantly, ED-1 resulted in sugar yields of 88.88 ± 3.64 to 106.86 ± 1.21 g L^{-1}, sufficient to achieve ethanol titers ≥40 g L^{-1}, the threshold required for industrial relevance. A semi-integrated bioprocess validated this outcome, producing 42.09 ± 2.38 g L^{-1} ethanol and an estimated yield of 213.38 L of ethanol per dry ton of pretreated biomass, requiring only 20.83 L of cellulase and 6.25 L of hemicellulase per ton. Remarkably, these enzyme dosages were approximately tenfold lower than those reported in prior studies.

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1. This study quantitatively assesses the high-performance pre-commercial cellulase and hemicellulase loadings for the hydrolysis of structural carbohydrates in diverse bioenergy feedstocks, achieving an approximately tenfold reduction in enzyme loading.
2. The enzyme optimization strategies enabled the standardization of enzyme dosages to 20.83 L of cellulase and 6.25 L of hemicellulase per ton of lignocellulosic feedstocks, while maintaining high sugar yields sufficient to meet industrial ethanol titer thresholds without compromising ethanol yield, as demonstrated through a semi-integrated bioprocess.
3. The optimized enzyme framework is promising for a potential lignocellulosic biorefinery; however, scale-up studies are still needed to validate the performance of the selected enzyme loading, as well as the semi-integrated bioprocess. Additionally, the fermentation rate could be improved to facilitate faster xylose consumption by using corn steep liquor as a suitable nutrient source instead of urea.

1 Introduction

The rapid increase in global energy demand, coupled with the depletion of fossil fuel reserves, necessitates the exploration of alternative renewable energy sources to address future energy challenges. Petroleum-based fuels, derived from crude oil, remain the dominant non-renewable energy source in the transportation sector and are significant contributors to greenhouse gas emissions. Blending gasoline with ethanol, an oxygenated fuel, reduces greenhouse gas emissions.[1] Ethanol is one of the alternative fuels for the transportation sector, as it was used as a transportation fuel in the Second World War (WWII) due to the shortage of crude oil supply.[2] Ethanol production from starch and sucrose-based agricultural feedstocks is referred to as first-generation (1G) bioethanol, a well-established and commercialized industrial process.[3] Brazil and the USA are the two most significant producers of 1G bioethanol from sugarcane and corn, respectively.[4] Countries such as India, China, Europe, and Canada also produce 1G bioethanol using various agricultural feedstocks.[5] However, producing

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bioethanol from edible sources would impede food security in the future. Instead, inedible agricultural crop residues, such as rice straw, wheat straw, sugarcane bagasse, sorghum stalks, and corn stover, can be used as feedstocks for bioethanol production.[5] Bioethanol produced from agricultural crop residues is referred to as second-generation (2G) bioethanol. It has dual benefits: it does not compete with food and feed and can reduce greenhouse gas emissions from stubble burning.

Considerable progress has been made in deconstructing lignocellulosic biomass to reduce the recalcitrance of lignin, thereby facilitating the effective hydrolysis of structural carbohydrates. Dilute acid, alkali, ionic liquids (ILs), natural deep eutectic solvents (NADESs), and hydrothermal pretreatments are the most widely used deconstruction techniques for lignocellulosic biomass.[6,7] Among them, hydrothermal pretreatment is one of the promising approaches for deconstructing lignocellulosic biomass with a negligible concentration of sugar decomposition products (furans, formic acid, and levulinic acid), which eventually helps in enhancing the ethanol yield in the subsequent fermentation step.[8] Hydrothermal pretreatment eliminates the need for washing and buffering agents before the enzymatic hydrolysis and detoxification steps before fermentation.[8] Unlike dilute acid, dilute alkali, IL, and NADES pretreatments, hydrothermal pretreatment-based lignocellulosic biorefineries do not require solid–liquid separation and solvent/catalyst recovery (ionic liquid and eutectic) unit operations.[9–12] Previous studies have demonstrated that hydrothermal pretreatment of various bioenergy feedstocks yields higher ethanol titers, yields, and productivity by substituting an inexpensive nitrogen source, urea, for yeast extract and peptone during fermentation.[8] Except for 0.7 kg of NaOH per ton of pretreated biomass (pH adjustment for fermentation), no other chemicals are needed in hydrothermal pretreatment-based lignocellulosic biorefineries, indicating that by-products generated in the downstream process do not require a specialized waste treatment section.

However, the cost of the enzyme for lignocellulosic biorefineries is a significant barrier, accounting for 30% of the operating cost.[13] The enzyme cost contributed $0.38 per L of ethanol in the dilute acid pretreatment-based lignocellulosic biorefinery.[14] Published reports estimate that the cost contribution of enzymes to lignocellulosic bioethanol production varies significantly, including $0.02 per L, $0.07 per L, $0.08 per L, $0.09 per L, and 0.11 per L depending on many factors like the type of pretreatment, choice of feedstock, enzyme loading, hydrolysis time, ethanol yield, and type of wastewater treatment plant to treat the effluent.[14] It is assumed that the enzyme cost would be reduced with technological innovations in enzyme production and advancements in the conversion process of lignocellulosic biomass.[15] With limited unit operations, no usage of chemical agents, and negligible efforts on effluent treatment, the hydrothermal pretreatment-based biorefinery is one of the leading lignocellulosic biomass conversion technologies. Optimization of enzyme dosage across diverse bioenergy feedstocks pretreated in pilot-scale continuous hydrothermal reactors has not yet been systematically investigated. Previous studies have reported enzyme loadings ranging from 0.17 to 0.21 mL of cellulase and 0.04 to 0.05 mL of hemicellulase per gram of hydrothermally pretreated substrate.[16–20] When normalized to an industrial scale, these dosages correspond to approximately 170–210 L of cellulase and 40–50 L of hemicellulase per ton of biomass. The variability in enzyme loading across studies hampers accurate techno-economic analysis of lignocellulosic biorefineries, making it challenging to establish realistic benchmarks for process feasibility and cost reduction.

Standardizing the enzyme loading on different bioenergy feedstocks for maximizing the hydrolysis of cellulose and xylan, along with the focus on attaining substantial sugar titers to achieve the industrial ethanol titer threshold of >40 g L^{-1} (ref. 21 and 22) in subsequent fermentation, will allow cost-effective production of 2G ethanol. In this study, pilot-scale continuous hydrothermally pretreated feedstocks, oilcane, energycane, sorghum, and miscanthus were evaluated to optimize the loadings of pre-commercial cellulase (NS22257) and hemicellulase (NS22244) for maximizing sugar yields. In addition, ethanol yields at specific enzyme dosages were estimated, providing a framework to refine cost analyses as improved data become available.

2 Materials and methods

2.1. Feedstock collection and processing

Four bioenergy feedstocks, namely, oilcane, energycane, sweet sorghum, and *Miscanthus x giganteus*, were used in this study. Energycane and oilcane were collected from experimental fields (latitude: 29.40879° N, longitude: 82.17119° W) of the University of Florida, Gainesville. Sweet sorghum and miscanthus were collected from the Energy Farm (latitude: 40.06604° N, longitude: 88.20836° W) at the University of Illinois, Urbana-Champaign. Sweet sorghum, oilcane, and energycane stems were processed using a commercial juicer (Edwards Engineering Corp., SCM-APL-3C, IL), followed by using a fiber press (Vincent Corporation, CP-4, Tampa, FL) to extract the juice. The leftover bagasse was washed on a Vibro Energy Separator (SWECO, HX48S686CBTL, Florence, KY) with city water to remove residual sugars and then dried overnight at 48 °C in a tray dryer. The dried bagasse samples were hammer milled to reduce the particle size to 1–3 cm, while the grassy feedstock, *Miscanthus x giganteus*, was processed using a hammer mill (Schutte-Buffalo Industrial Hammer Mill, W-8-H, Buffalo, NY). Compositional analysis of sweet sorghum bagasse was performed according to the NREL procedures,[23] while the compositional data of miscanthus, oilcane, and energycane have been previously reported.[8]

2.2. Pilot-scale hydrothermal pretreatment

A pilot-scale continuous hydrothermal pretreatment reactor (SüPR·2G Reactors, Advance Bio Systems LLC, Milford, OH) was used to deconstruct the lignocellulosic conglomerate matrix of sweet sorghum, oilcane, energycane, and mis-

canthus. Before hydrothermal pretreatment, the lignocellulosic biomass samples were rehydrated to 50% (w/w) in a ribbon blender. Bagasse samples of sweet sorghum, oilcane, and energycane were hydrothermally pretreated at 190 °C with 10 bar pressure for 10 min, while the miscanthus was pretreated at 170 °C with 7.5 bar pressure for 10 min.[8,18,19,24,25] The pretreated solids were dried overnight at 48 °C and then disc milled (Quaker City Grinding Mills, Pottstown, PA; Model 4E) to further reduce the particle size. Compositional analysis of hydrothermal pretreated energycane, sweet sorghum, and miscanthus was performed according to the NREL procedures,[23] and oilcane composition was previously reported.[8] The compositional data, such as cellulose, hemicellulose, and acid-insoluble lignin (AIL) of selected bioenergy feedstock samples, are shown in Table 1. Acid-soluble lignin was not reported due to interference from furfural and 5-HMF during UV-Vis spectroscopy.[8]

2.3. Optimization of enzyme loadings

Fed-batch enzymatic hydrolysis of pretreated samples was conducted in a 250 mL screw-cap conical flask containing 12 g of biomass (oven-dried weight, OWD) in 40 mL of liquid medium, corresponding to a 30% (w/v) solid loading. Biocatalysts, cellulase (NS22257), and hemicellulase (NS22244) were loaded based on their protein concentrations, determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce BCA protein assay kit, Thermo Fisher Scientific, USA), which contains a protein concentration of 379.57 ± 18.83 mg mL^{-1} cellulase and 232.02 ± 3.16 mg mL^{-1} hemicellulase. To optimize the enzyme dose, cellulase protein concentrations varied at 20 mg, 40 mg, and 60 mg g^{-1} cellulose, corresponding to the hemicellulase protein concentrations of 7.25 mg, 14.5 mg, and 21.75 mg g^{-1} xylan. The combinations of cellulase and hemicellulase doses were referred to as ED-1 (20 mg and 7.25 mg), ED-2 (40 mg and 14.5 mg), and ED-3 (60 mg and 21.75 mg). Initially, 4 g of biomass was loaded at 0 h, and the remaining 8 g of biomass was divided into equal amounts and added at 3 h and 6 h intervals to achieve 30% (w/v) solid loading. An enzymatic hydrolysis reaction was performed at 50 °C for 72 h with agitation at 185 rpm in a shaking incubator. An aliquot of 0.6 mL was withdrawn before adding solids and centrifuged at 8600 rpm for 10 min, followed by filtration of the supernatant with a 0.2 μm syringe filter to quantify sugars by high-performance liquid chromatography (HPLC). Cellulose and xylan hydrolysis efficiencies were calculated according to a previous study.[8]

2.3.1 Scale-up of enzymatic hydrolysis. Fed-batch enzymatic hydrolysis of hydrothermally pretreated sweet sorghum bagasse was conducted in a 75 L bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ) with a working volume of 52 L at a protein concentration of 40 mg of cellulase and 14.5 mg of hemicellulase (ED-2) per g of cellulose and xylan, respectively. Initially, 35 L of distilled water was loaded into the reactor, followed by 500 mL of cellulase and 150 mL of hemicellulase. The final liquid volume was made to 40 L, and the impeller speed was set to 150 rpm. Once the liquid medium reached the set point of 50 °C, 4 kg of pretreated solids (ODW) were added to the reactor at 21 min, representing a 10% (w/v) solid loading. The additional solids were added at 221 min and 421 min to achieve 20% and 30% (w/v) solid loading, with the corresponding impeller speeds increased to 275 rpm and 325 rpm. It took 20 min to load 4 kg of solids into the reactor each time. An aliquot of 1 to 1.5 L of enzymatic slurry was withdrawn in three containers (~350 to 500 mL each) before each addition of pretreated solids. Finally, an aliquot of 50 mL of the enzymatic slurry was collected at 12 h and then harvested at 16 h. Samples were centrifuged at 8600 rpm for 10 min to collect the hydrolysate for sugar quantification. The pH of the hydrolysis medium was monitored to ensure it remained above 3. Continuous monitoring of the enzymatic hydrolysis process parameters, such as changes in bioreactor weights, pH, temperature, and impeller speed, with the corresponding time, and the data are provided in Fig. 1.

2.4. Semi-integrated bioprocess for ethanol production

Hydrothermal pretreated sweet sorghum bagasse was used as a model substrate for the semi-integrated bioprocess, an integrated approach for enzymatic hydrolysis and subsequent fermentation in a single unit.[8] Fed-batch enzymatic hydrolysis was performed at 25% (w/v) solid loading with ED-1 for 18 h at 50 °C with 186 rpm. Enzymatic hydrolysis was initiated with 10% (w/v) solid loading, and additional solids were added at 3 h to increase the loading to 20% (w/v). The final dosage of solids was added at 6 h. After 18 h, the reaction was cooled to 30 °C, and 4 g L^{-1} urea was added to the enzymatic slurry. The pH was adjusted between 5.6 and 5.8 using 5 M NaOH and agitated at 186 rpm for 2 h to monitor for any pH drop. 1 mL of seed culture (an initial cell concentration of 1.7 mg per 50 mL)

Table 1 Compositional analysis of hydrothermal pretreated feedstocks[a]

Feedstock	Extractives (%)	Cellulose (%)	Xylan (%)	Arabinan (%)	Acetic acid (%)	AIL (%)
Raw biomass						
Sweet sorghum	16.56	41.05 ± 1.30	15.79 ± 0.93	0.30 ± 0.02	3.93 ± 0.11	15.98 ± 0.51
Hydrothermal pretreatment followed by mechanical disc refining						
Sweet sorghum	NA	40.30 ± 0.24	17.64 ± 0.23	1.18 ± 0.01	3.67 ± 0.01	18.27 ± 0.16
Miscanthus	NA	37.59 ± 0.05	18.82 ± 0.12	1.49 ± 0.01	3.41 ± 0.02	17.14 ± 1.02
Energycane	NA	41.27 ± 0.23	19.17 ± 0.11	1.22 ± 0.01	3.36 ± 0.18	15.38 ± 1.16
Oilcane[8]	NA	40.57 ± 0.87	23.62 ± 0.49	1.17 ± 0.01	4.47 ± 0.05	12.64 ± 0.44

AIL, acid-insoluble lignin; NA, not applicable. [a] Average ± standard deviation. The composition of oilcane was represented from a previous study.[8]

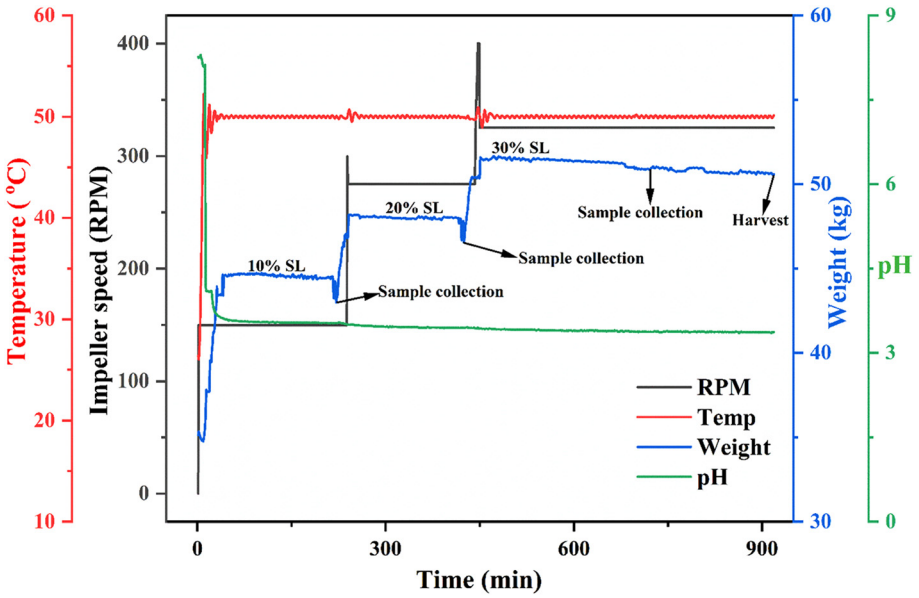

Fig. 1 Real-time monitoring of enzymatic hydrolysis data for the pH, temperature, bioreactor weights, and impeller speed, with the corresponding time using a scale-up approach.

of xylose-fermenting engineered *Saccharomyces cerevisiae* was added to the enzymatic slurry to initiate the fermentation for 144 h. The seed culture was cultivated in YPDX (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 12 g L^{-1} glucose, and 8 g L^{-1} xylose) medium at 30 °C with 140 rpm for 18 h. Aliquots were collected at 24 h intervals to quantify residual sugars and ethanol by HPLC.

2.5. High-performance liquid chromatography

Quantification of glucose, xylose, and ethanol was determined by using an HPLC system (Waters e2692 Separation Module, Waters Corporation, Milford, MA, USA) equipped with a 2414 refractive index detector and an Aminex HPX-87H column (300 × 7.8 mm, 9 µm particle size; Bio-Rad Laboratories, Hercules, CA, USA). Temperatures of the RI and column were maintained at 30 °C and 65 °C, respectively, to separate the compounds using 5 mM H_2SO_4 as the eluent with a flow rate of 0.6 mL min^{-1}.

2.6. Statistical analysis

The enzyme dosage optimization experiments were performed in triplicate, and bioethanol production runs were conducted in duplicate. Means and standard deviations are reported. Regression analysis was conducted for each enzyme dosage to evaluate the proportional relationship between the sugar yield increments at the corresponding solid loadings. Regression analysis was selected for sugar yield increments because it effectively identifies the linear relationship between multiple input variables, such as enzyme dosage and solid loading, and the resulting sugar yield. Tukey's *post hoc* test was performed following a significant ANOVA to identify similarities in sugar yields across different enzyme dosages and to conduct pairwise comparisons among the selected bioenergy feedstocks. Tukey's *post-hoc* test is the preferred test for pairwise comparisons of group means, such as all-pairwise comparisons of sugar yield across different enzyme dosages and selected bioenergy feedstocks, rather than only examining the effect of enzyme dosages on sugar yield. Tukey's test and regression analysis were performed using OriginPro (Version 2023, OriginLab Corporation, Northampton, USA).

3 Results and discussion

3.1. Effect of enzyme loading on sugar yields

Producing industrial ethanol titers (≥40 g L^{-1}) from lignocellulosic biomass is one of the essential criteria for a 2G biorefinery, enabling an economically viable distillation process.[21,22] Previous studies have reported that achieving 120 to 140 g L^{-1} of fermentable sugars (glucose and xylose) requires a 30% (w/v) solid loading of various hydrothermally pretreated bioenergy feedstocks.[8] These sugar concentrations are sufficient to achieve the threshold titers of industrial ethanol, ≥40 g L^{-1}; thus, the enzyme optimization study was conducted at 30% solid loading. With the combination of 20 mg of cellulase and 7.25 mg of hemicellulase protein concentrations (ED-1), 106.86 ± 1.21 g L^{-1}, 102.53 ± 2.24 g L^{-1}, 98.58 ± 3.88 g L^{-1}, and 88.88 ± 3.64 g L^{-1} sugars (glucose and xylose) were attained at a 30% (w/v) solid loading of OC, SS, MC, and EC, respectively (Fig. 2). The sugar concentrations were slightly increased to 130.37 ± 1.09 g L^{-1}, 123.78 ± 1.54 g L^{-1}, 108.05 ± 6.60 g L^{-1}, and 105.04 ± 1.12 g L^{-1} by using the protein concentration of 40 mg of cellulase with 14.5 mg of hemicellulase (ED-2) for OC, SS, EC, and MC (Fig. 2). The sugar increments ranged between 6.46 g L^{-1} and 23.51 g L^{-1} by increasing the enzyme dosage from ED-1 to ED-2. At 60 mg

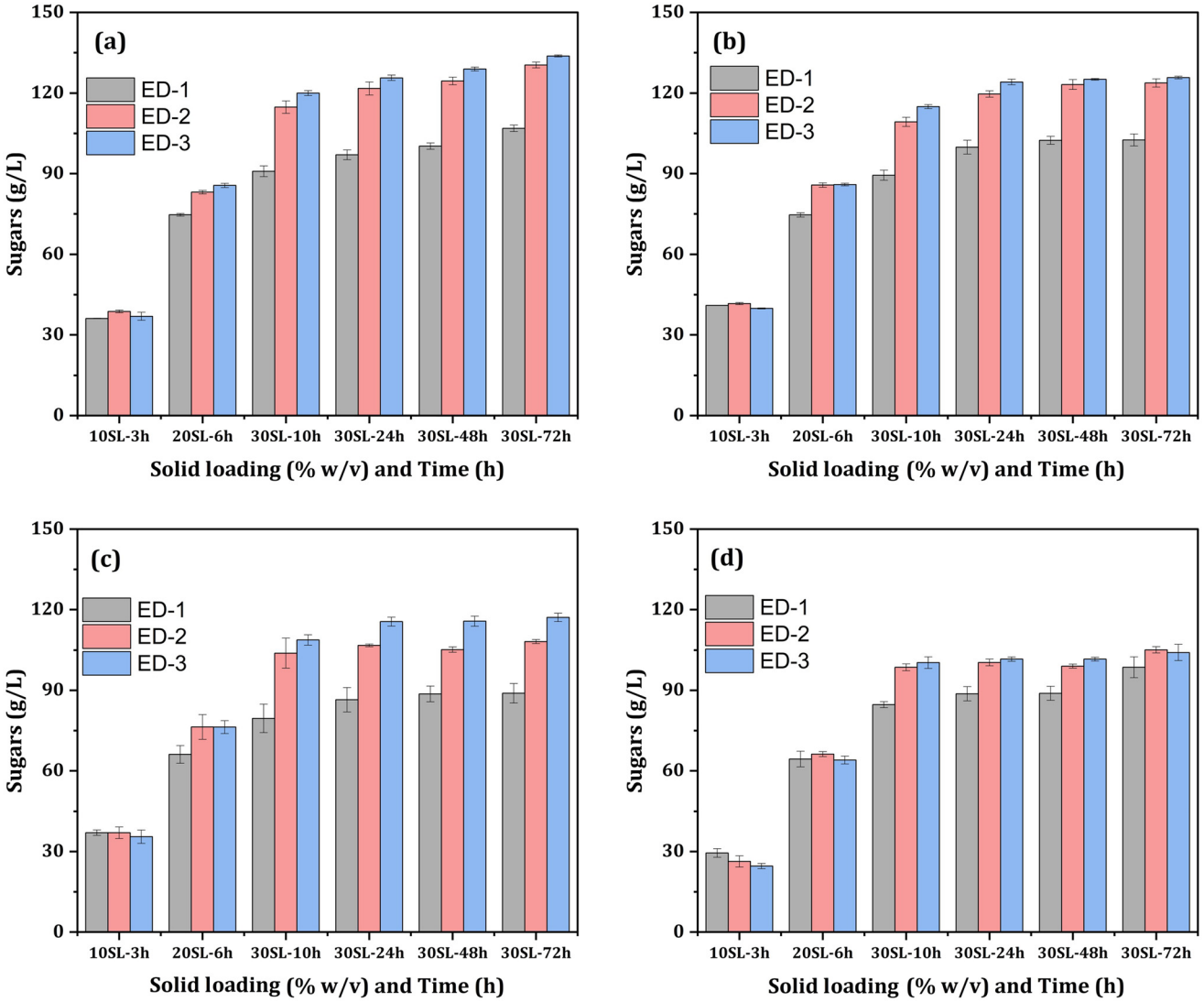

Fig. 2 Sugar yields (glucose and xylose) from (a) oilcane, (b) sweet sorghum, (c) energycane, and (d) miscanthus, corresponding to time and solid loadings at different enzyme dosages (ED-1, ED-2, and ED-3).

of cellulase protein with 21.75 mg of hemicellulase protein (ED-3), 133.72 ± 0.36 g L^{-1}, 125.76 ± 0.46 g L^{-1}, 117.07 ± 1.57 g L^{-1}, and 104.046 ± 2.95 g L^{-1} sugar yields were attained from OC, SS, EC, and MC, respectively (Fig. 2), accounting for 1.98 to 9.02 g L^{-1} sugar yield increment compared to ED-2. As shown in Fig. 3, Tukey's *post hoc* test confirmed that sugar yields obtained with ED-2 and ED-3 were not significantly different for oilcane (OC), sweet sorghum (SS), and miscanthus (MC) ($p < 0.05$). In contrast, for energycane (EC), the 9.02 g L^{-1} increase in sugar yield between ED-2 and ED-3 was sufficient to result in a statistically significant difference. Notably, sugar yields for MC were not significantly different across any of the enzyme dosages evaluated (ED-1, ED-2, or ED-3) (Fig. 3). This behavior is attributed to the hydrothermal pretreatment temperature of 170 °C, which is insufficient to effectively deconstruct the lignocellulosic matrix of MC, thereby limiting enzyme accessibility, particularly to cellulose, and constraining sugar release even when enzyme loadings were increased two- to three-fold. Sugar yields obtained with ED-1 for SS and OC, as well as ED-2 for EC, were statistically comparable to those observed for MC across all enzyme dosages. This outcome reflects both the pretreatment severity and the higher cellulose content of SS, EC, and OC (Table 1), which likely resulted in higher sugar yields at lower enzyme dosages relative to MC (Fig. 3).

ED-2 can be considered an optimum enzyme dosage for hydrothermal pretreated lignocellulosic feedstocks. To validate this, a regression analysis (R^2) was conducted for sugar yield increments corresponding to each addition of biomass solid loading in the enzymatic hydrolysis, and the linear fit results were similar to those of the ED-3 regression analysis. ED-2 and ED-3 exhibited a linear fit with $R^2 \geq 0.99$ for OC, MC, EC, and SS (Fig. 4), indicating that the sugar yields were directly proportional to the biomass solid loadings. For example, in OC hydrolysis using ED-2, sugar yields were 38.69 ± 0.51 g L^{-1},

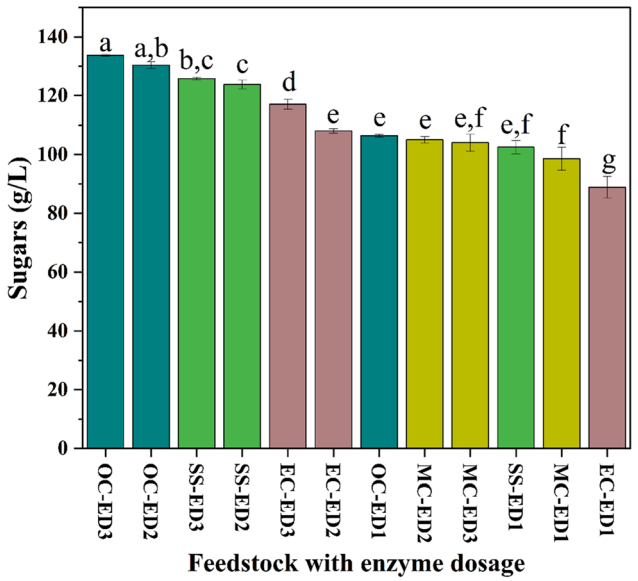

Fig. 3 Sugar yields of oilcane (OC), sweet sorghum (SS), energycane (EC), and miscanthus (MC) at different enzyme dosages: ED-1, ED-2, and ED-3. Statistical analysis was performed using one-way ANOVA, followed by Tukey's test. The bar sharing the same letter indicates that there was no significant difference in the sugar yields.

83.06 ± 0.65 g L^{-1}, and 130.37 ± 1.09 g L^{-1} at 10%, 20%, and 30% solid loading, accounting for 43.45 ± 4.38 g L^{-1} sugar yield increment for each 10% solid increment.

3.2. Effect of cellulase and hemicellulase loadings on glucose and xylose yields

With ED-3 (a combination of 60 mg of cellulase and 21.75 mg of hemicellulase), cellulose hydrolysis efficiencies accounted for 60.67 ± 0.81% to 75.02 ± 0.31% (Fig. 5). Among the selected bioenergy feedstocks, the highest cellulose hydrolysis efficiency was observed in SS, with a glucose yield of 82.46 ± 0.35 g L^{-1} (Fig. 6a). No significant difference ($p < 0.05$) in cellulose hydrolysis efficiencies was observed between OC (62.82 ± 0.07%), MC (62.58 ± 1.17%), and EC (60.67 ± 0.81%). Still, the glucose yield of MC (59.24 ± 1.68 g L^{-1}) was lower than those of EC (68.17 ± 0.91 g L^{-1}) and OC (69.51 ± 0.08 g L^{-1}). Hydrothermal pretreatment of MC was conducted at 170 °C instead of 190 °C to avoid the degradation of anthocyanins (a value-added natural colorant), which could be a significant reason for the lowest glucose yield in MC compared to all other feedstocks. Additionally, the lower cellulose content in MC is another reason for the lowest glucose yield (Table 1). Xylan hydrolysis reached more than 87% for all feedstocks (Fig. 5). Unlike crystalline cellulose, xylan is an amorphous polymer that is easily depolymerized at lower temperatures than cellulose.[3] Based on the thermogravimetric analysis, xylan thermal degradation occurs between 160 °C and 320 °C, with a maximum temperature (T_{max}) of 298 °C. In contrast, crystalline cellulose degradation occurs between 280 °C and 360 °C, with a T_{max} of 335 °C.[26,27] Therefore, xylan tends to have a higher rate of hydrolysis than cellulose.

Across all enzyme loadings, the highest cellulose hydrolysis was observed in SS (Fig. 4b). The highest xylan hydrolysis was observed in OC (Fig. 5a). For the selected bioenergy feedstocks, cellulose hydrolysis increment was less than 5% upon increasing the cellulase protein concentration from 40 mg to 60 mg g^{-1} cellulose (Fig. 5). On the other hand, 14.5 mg of hemicellulase protein concentration was considered optimal for xylan hydrolysis, since no significant ($p < 0.05$) increment in xylan hydrolysis at 21.75 mg of hemicellulase protein concentration was observed. Negligible increases in glucose (<8.2 g L^{-1}) and xylose (<1 g L^{-1}) yields at ED-3 (Fig. 5) provide the principal evidence for considering ED-2 as the optimized enzyme dosage for the selected bioenergy feedstocks.

Hydrothermal pretreatment is an effective method for deconstructing the lignocellulosic matrix of grassy feedstocks and supports substantial sugar yields in subsequent enzymatic hydrolysis. The optimized enzyme dosages identified in this study may be applicable to other members of the grass family (*Poaceae*), including corn stover, wheat straw, rice straw, and switchgrass. However, these dosages require validation across different pretreatment techniques, as methods such as ammonia fiber expansion (AFEX) and ionic liquid pretreatments are more effective at lignin solubilization than hydrothermal pretreatment. For instance, sugar yields from agave bagasse vary significantly depending on the pretreatment method, with AFEX, ionic liquid, and hydrothermal processes yielding 42.6 g, 39.7 g, and 26.9 g per 100 g of biomass, respectively.[28] Additionally, woody biomass needed further validation, as it is more recalcitrant than grassy feedstocks and may require different pretreatment approaches to reduce recalcitrance, enabling effective hydrolysis of structural carbohydrates with optimized enzyme dosages. For example, poplar biomass needs harsher AFEX pretreatment conditions than corn stover to achieve equivalent sugar yields upon enzymatic hydrolysis.[29] Ionic liquids are also effective in reducing the recalcitrance of woody biomass; however, pretreatment severity varies with biomass recalcitrance.[30] Therefore, the optimized enzyme dosage must be further validated for highly recalcitrant woody substrates such as walnut, poplar, eucalyptus, and almond.

3.2.1 Scale-up of enzymatic hydrolysis with ED-2. The decrease in distilled water pH to 4.11 following the addition of cellulase and hemicellulase is attributed to the intrinsic pH of the enzyme preparation, as their optimal activity typically occurs within a pH range of 4 to 5.[31] The subsequent addition of 4 kg of solids further reduced the pH of the hydrolysis medium to 3.61. With each 10% increment in solid loading, the pH of the hydrolysis medium decreased to 3.51 and 3.44, eventually stabilizing at 3.37 until harvest. The decrease in the pH during enzymatic hydrolysis was consistent with our previous findings.[8] The observed pH reduction during enzymatic hydrolysis is primarily due to the release of acidic compounds. Hemicellulose hydrolysis generates xylose, arabinose, and acetic acid,[32] with acetyl group cleavage contributing significantly to acidification. Additionally, lignin-derived phenolic acids further lower the pH. For instance, 4-hydroxybenzoic

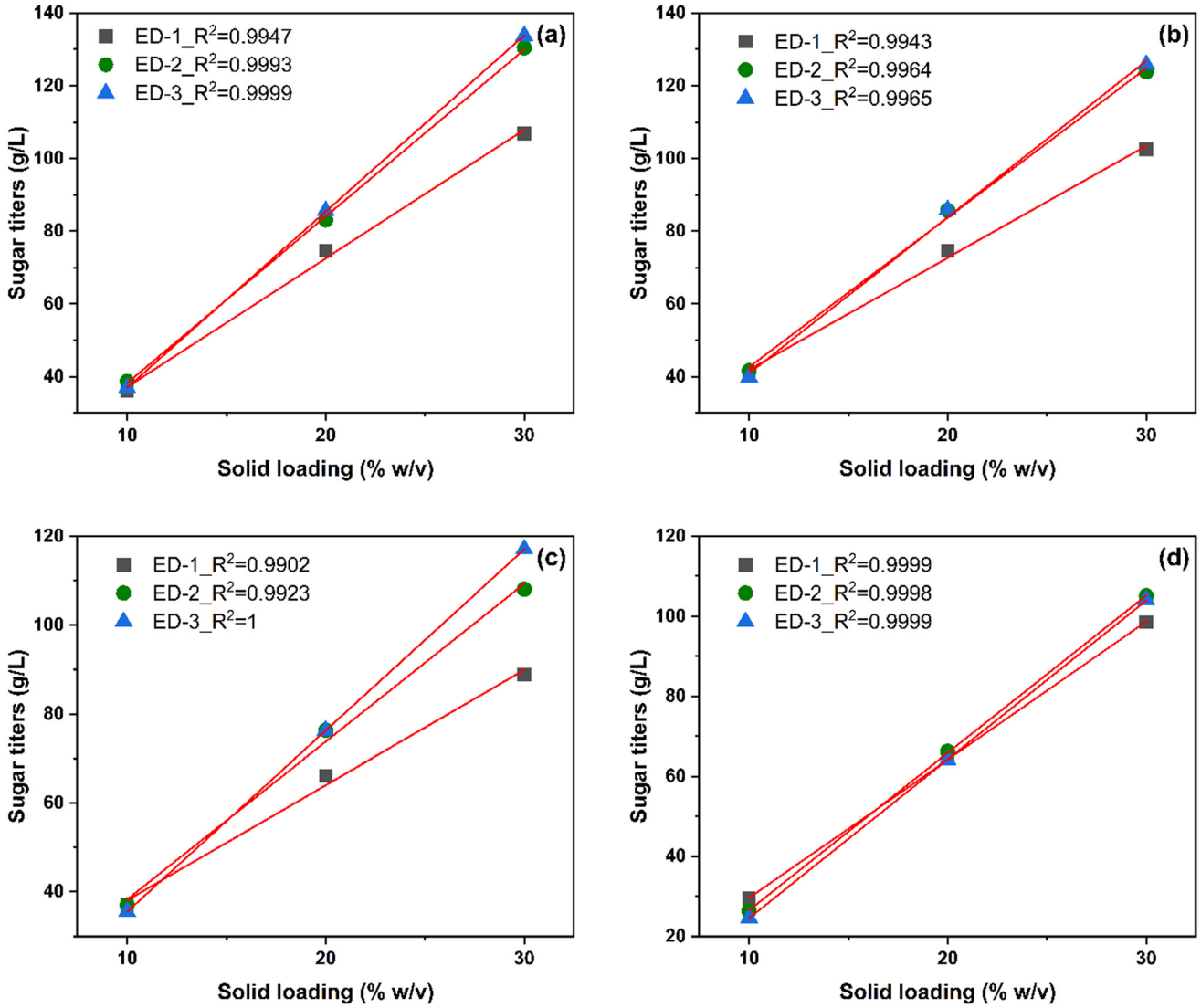

Fig. 4 Regression analysis of the sugar yield for the solid loadings at different enzyme dosages: (a) oilcane, (b) sweet sorghum, (c) energycane, and (d) miscanthus.

acid, a common phenolic compound in lignocellulosic biomass, is released during hydrothermal pretreatment and persists through the enzymatic hydrolysis.[8] Despite a reduced pH, hydrolysis efficiency was not adversely affected. At 30% (w/v) solid loading, 75.56 ± 0.93% cellulose and 99.03 ± 0.68% xylan were converted to 83.05 ± 1.02 g L^{-1} glucose and 47.64 ± 0.33 g L^{-1} xylose. These values were slightly higher than those reported in shake flask studies of sweet sorghum bagasse (Fig. 6), likely due to improved mixing in the bioreactor. To evaluate slurry handling and sampling consistency, 1–1.5 L of enzymatic slurry was withdrawn into three containers to assess flowability through the sample port and uniformity of sugar titers. Efficient mixing of slurry at high solids loading is critical, as poor mixing can reduce enzymatic hydrolysis efficiency, clog the sampling ports and lead to inaccurate sugar measurements.[30] Approximately 1.5 L of slurry was collected within 10 minutes (9 L h^{-1}) at 20% (w/v) solid loading (7.2 h), yielding consistent sugar concentrations of 69.51, 70.35, and 70.79 g L^{-1} across samples, indicating uniform hydrolysis at scale. At the bioreactor scale, 49.93 kg of slurry was recovered from 50.58 kg, achieving 98.7% recovery. The hydrolysate contained 130.69 ± 1.35 g L^{-1} total sugars, including 83.05 ± 1.02 g L^{-1} glucose and 47.64 ± 0.33 g L^{-1} xylose. The combination of high sugar yields within 16 h and effective mixing without clogging demonstrates that ED-2 is a suitable candidate for successful scale-up of enzymatic hydrolysis.

3.3. ED-1 has the potential for lignocellulosic biorefineries

Notably, xylan hydrolysis exceeded 75% with 7.25 mg of hemicellulase protein concentration, and xylose yield increments ranged from 2.86 to 10.16 g L^{-1} by increasing the hemicellulase protein concentration from 7.25 to 14.5 mg (Fig. 6b).

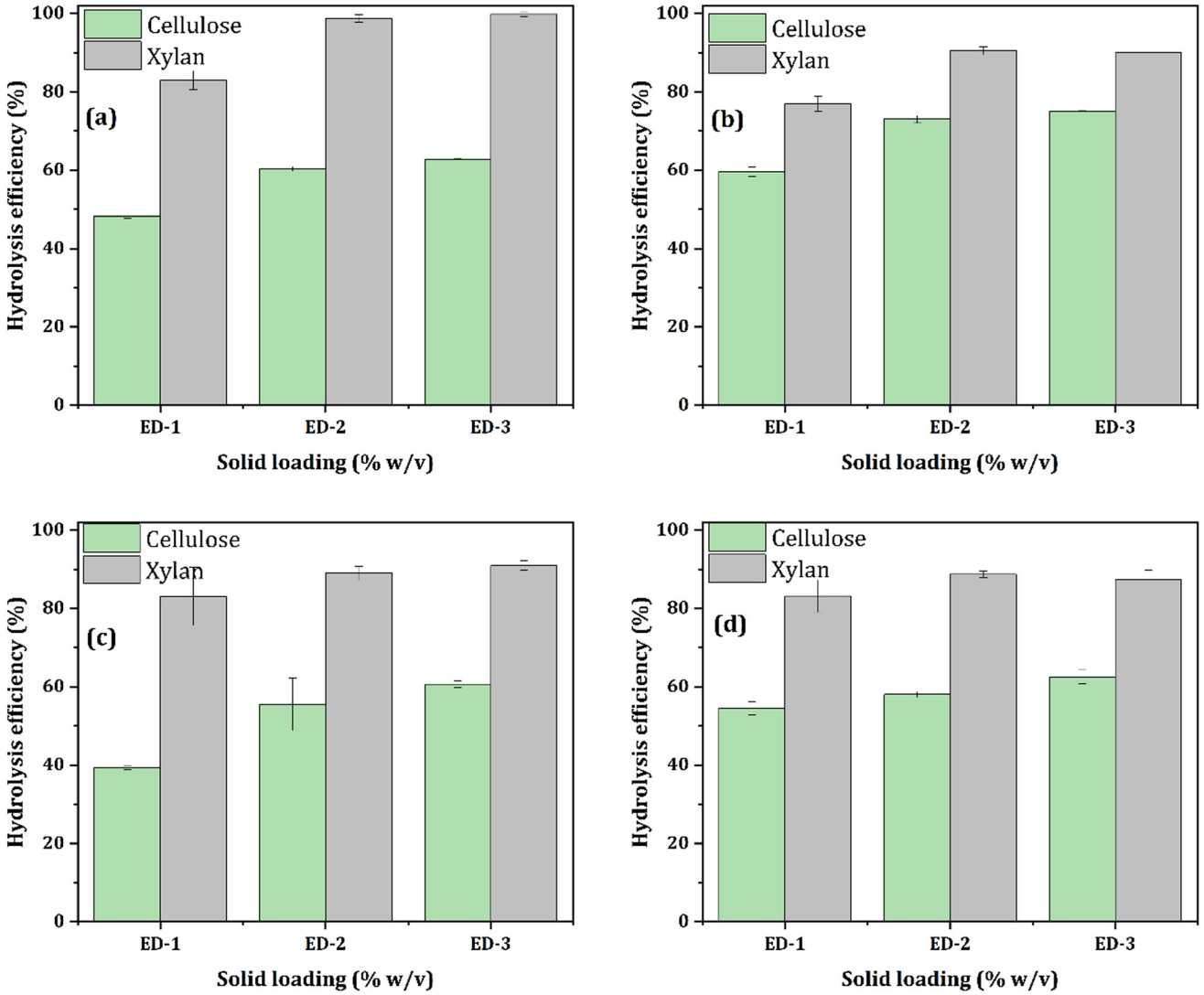

Fig. 5 Cellulose and xylan hydrolysis efficiencies at 30% (w/v) solid loading of (a) oilcane, (b) sweet sorghum, (c) energycane, and (d) miscanthus at selected enzyme loadings.

Therefore, 7.25 mg of hemicellulase protein loading can also be ideal for xylan hydrolysis. Similarly, 20 mg of cellulase protein loading would also be an excellent choice for the bioenergy feedstocks because the glucose yield increments ranged from 3.59 to 15.74 g L^{-1} upon increasing the cellulase protein concentration two-fold (Fig. 6a). Since the cost of enzymes is one of the significant barriers for lignocellulosic biorefineries, ED-1 (20 mg of cellulase protein and 7.25 mg of hemicellulase protein) is the best candidate for enzymatic hydrolysis, resulting in 88.88 ± 3.64 to 106.86 ± 1.21 g L^{-1} sugar concentration and an ethanol concentration ≥40 g L^{-1}. Additionally, regression analysis of sugar yields between 10% and 30% (w/v) with ED-1 demonstrated a linear fit with $R^2 \geq 0.99$ (Fig. 4), providing compelling statistical support to validate that ED-1 can potentially improve sugar yields by increasing solid loading.

Theoretically, the sugar yields achieved with ED-1 could result in ethanol titers ranging from 48.08 ± 1.85 to 57.25 ± 0.61 g L^{-1} (Table 2). Similarly, an ethanol titer of 59.33 ± 0.57 to 72.24 ± 0.55 g L^{-1} and 61.85 ± 1.51 to 76.98 ± 0.18 g L^{-1} could be produced from the sugar yields obtained at ED-2 and ED-3, respectively. The detailed ethanol titer increments corresponding to the enzyme dosages (ED-1 to ED-3) are shown in Table 2. From an economic perspective, increasing the enzyme dosage to threefold for a maximum increment of ~20 g L^{-1} ethanol titer is not cost-effective. For example, the sugar concentrations obtained from the enzymatic hydrolysis of SS at ED-1, ED-2, and ED-3 have the potential to produce ethanol titers of 55.04 ± 1.14 g L^{-1}, 68.88 ± 0.78 g L^{-1}, and 72.92 ± 0.23 g L^{-1}, respectively. From a commercial standpoint, increasing the enzyme dosages to two- and threefold for ~14 g L^{-1} and ~18 g L^{-1} increments in ethanol titer is not ideal. When the process is scaled up, ED-1, ED-2, and ED-3 require 27.08 L (20.83 L of cellulase and 6.25 L of hemicellulase), 54.16 L (41.66 L of cellulase and 12.5 L of hemicellulase), and 81.24 L (62.49 L of cellulase and 18.75 L of hemicellulase) of enzyme cocktail (cellulase and hemicellulase at a 7.7 : 2.3 (v/v)

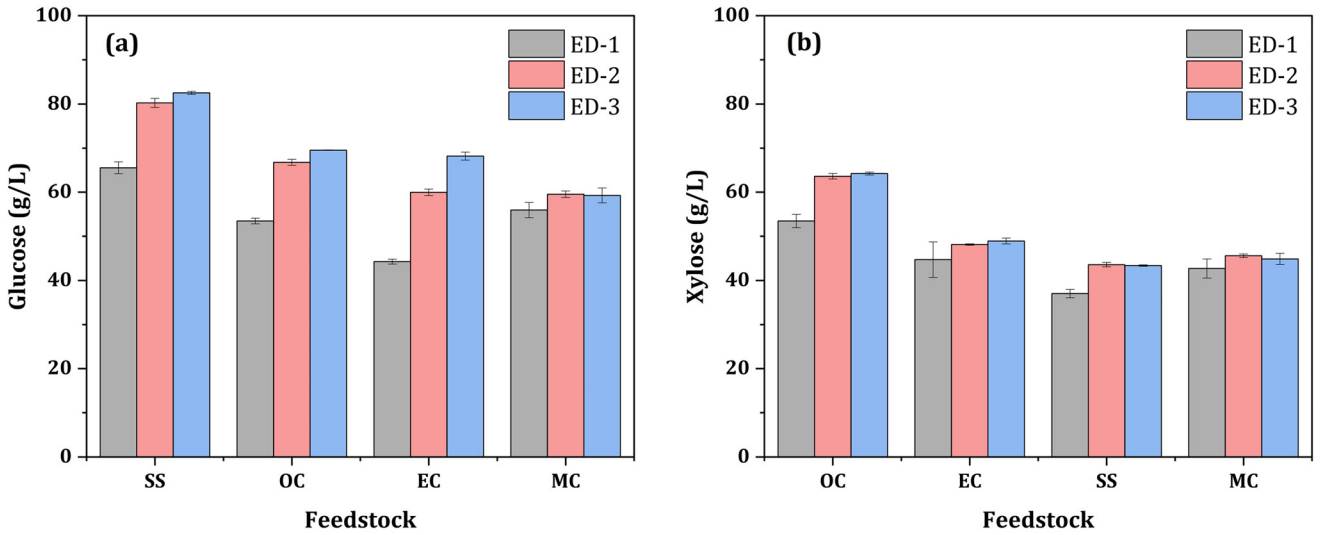

Fig. 6 Action of selected enzyme dosages for (a) glucose and (b) xylose yields.

Table 2 Theoretical ethanol titers from the enzymatic slurry of selected bioenergy feedstocks

	Theoretical ethanol titers[a] (g L^{-1})		
Feedstocks	ED-1	ED-2	ED-3
Oilcane	57.25 ± 0.61	72.24 ± 0.55	76.98 ± 0.18
Sweet sorghum	55.04 ± 1.14	68.88 ± 0.78	72.92 ± 0.23
Energycane	48.08 ± 1.85	61.97 ± 3.36	68.49 ± 0.81
Miscanthus	53.03 ± 1.98	59.33 ± 0.57	61.85 ± 1.51

[a] Theoretical ethanol titers were calculated by including the sugars that are present in cellulase and hemicellulase, as they are converted to ethanol during fermentation.

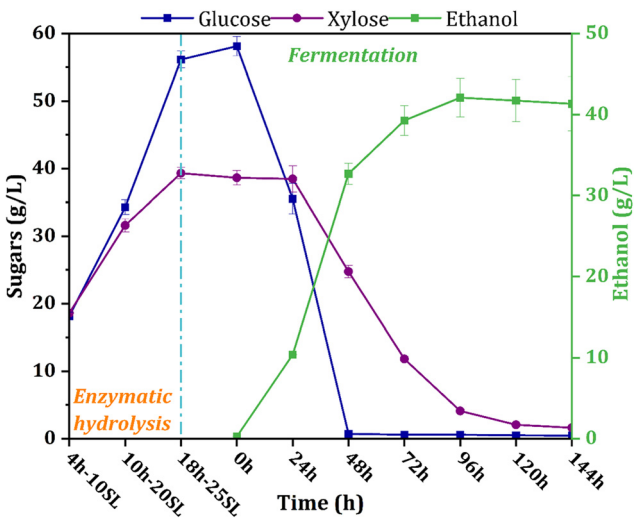

Fig. 7 Semi-integrated bioprocess of hydrothermal pretreated sweet sorghum bagasse for bioethanol production.

ratio), which can yield 232.53 L, 291.11 L, and 308.06 L of ethanol per dry ton (DT) of SS. The quantity of enzyme cocktail required for ED-3 (81.24 L) can hydrolyze the three tons of SS at the ED-1 (27.08 L) dose, eventually increasing the ethanol yield to 697.59 L instead of 308.06 L. Similarly, a 54.16 L enzyme cocktail can yield 465.06 L of ethanol from two tons of SS at an ED-1 dose. Therefore, using a lower enzyme dosage can improve the overall economics of the lignocellulosic biorefinery process.

Theoretical ethanol yield (232.53 L per ton) of SS with ED-1 was validated through the semi-integrated bioprocess, which yielded 96.75 g L^{-1} sugar (58.12 ± 1.42 g L^{-1} glucose and 38.63 ± 1.08 g L^{-1} xylose) with 25% (w/v) solid loading within 18 h and achieved an industrial ethanol titer of 42.09 ± 2.38 g L^{-1} at 96 h fermentation with engineered xylose fermenting yeast (Fig. 7). The ethanol yield accounted for 0.45 ± 0.01 g_p g_s^{-1}. According to the mass balance analysis, 250 kg of SS produced 53.34 L of ethanol, equivalent to 213.38 L of ethanol per ton of biomass. This represents a 19.14 L difference when comparing the theoretical ethanol yield of SS with the experimental value.

Ammonia fiber expansion (AFEX) also offers advantageous pretreatment techniques that enable effective lignin solubilization, reduce inhibitory compounds, and require minimal or no nutrient addition to fermentation, owing to residual ammonia and native plant-derived micronutrients in the recovered pretreated biomass.[33] In AFEX-pretreated corn stover hydrolysate fermentation, an ethanol titer of ~30 g L^{-1} with complete glucose consumption occurred at 24 h without nutrient supplementation. In comparison, adding 5 g L^{-1} yeast extract and 10 g L^{-1} peptone as nutrient supplements enhanced glucose consumption and ethanol productivity.[34] Additionally, nutrient supplementation enabled complete xylose consumption within 48 h of fermentation, whereas significant residual xylose persisted after 120 h without supplementation. However, the reported final ethanol titers did not differ significantly between nutrient-supplemented and non-supplemented conditions.[34] In one study, corn stover was

pretreated with AFEX (120 °C, 350 psi, and 30 min), compacted biomass with recycled ammonia (COBRA, 67 °C, 400 psi, and 327 min), and extractive ammonia (EA, 120 °C, 1300 psi, and 30 min). Fermentation of the resulting enzymatic hydrolysates yielded 34.6 g L^{-1}, 38.3 g L^{-1}, and 40.6 g L^{-1} ethanol, respectively, without nutrient supplementation.[12] Across all pretreatment methods, glucose was completely consumed within 18 h, whereas xylose required 96 h for near-complete utilization. The comparatively lower xylose conversion observed in COBRA-treated biomass was attributed to differences in nutrient composition. Specifically, the lower pretreatment temperature (67 °C) likely resulted in reduced release of plant-derived micronutrients compared to EA and AFEX, thereby limiting yeast metabolic activity.[12] Furthermore, in the absence of external nutrient supplementation, xylose fermentation generally requires greater nutrient availability than glucose fermentation.[12]

Pretreatment strategies can reduce or eliminate the need for nutrient supplementation; however, supplementation significantly enhances fermentation rates and shortens processing time. For instance, soaking in aqueous ammonia (SAA) pretreated switchgrass produced 0.11–48 g L^{-1} ethanol after 48 h without supplementation, likely due to lower acetic acid concentrations.[35,36] In contrast, drying SAA pretreated sugarcane bagasse enabled acetic acid removal as ammonium acetate, resulting in 94–100 g L^{-1} ethanol at 48 h with yeast extract and peptone supplementation.[32] Similarly, reductive catalytic fractionation (RCF) hydrolysates yielded 52.6 g L^{-1} ethanol (85.3%) at 60 h without supplementation but achieved the same yield in 25 h with yeast extract.[37] In a one-pot process using 10 wt% cholinium lysinate ([Ch][Lys]), ethanol titers (~25–27.6 g L^{-1} at 120 h) were unaffected by supplementation due to inhibitor presence; however, supplementation improved titers to 40–50 g L^{-1} under similar conditions.[30,32]

Although nutrients enhance microbial activity, their cost is critical for process viability. Low-cost supplements such as urea and corn steep liquor (CSL) are promising alternatives. Urea is widely used in the dry-grind ethanol industry to achieve up to 18% (v/v) ethanol within 48–72 h.[38] For lignocellulosic hydrolysates, which contain inhibitory compounds, CSL is particularly effective due to its rich nutrient profile.[39] Supplementation with 2.5 g L^{-1} CSL improved ethanol production and xylose consumption in AFEX-pretreated corn stover.[40]

In the RaBIT process, CSL addition (2.5 g L^{-1}) enhanced yeast activity at high cell density, reducing fermentation time from 120 h to 24 h while maintaining ≥40 g L^{-1} ethanol.[40] Shorter fermentation improves productivity and reduces energy demand. Consistently, urea supplementation achieved 39.2–42.09 g L^{-1} ethanol at 72–96 h, while similar titers may be attainable within 24 h using CSL, highlighting its potential for process intensification.

3.4. Enzyme volume per ton for cost calculation and the conventional process

It is essential to report a quantity of enzyme (kg or L) required per kg or dry ton (DT) of biomass or per gram of glucan to evaluate the accurate cost contribution of enzyme for 2G lignocellulosic biorefineries, as reporting the enzyme concentration in terms of mg protein per mL of enzyme and filter paper units has challenges in reproducing results and developing processes for commercialization. NREL has reported that the filter paper unit (FPU) assay is no longer reliable for measuring cellulase activity because it only converts 3.6% of the substrate, which is not a true lignocellulosic feedstock.[41] It has been recommended to estimate proteins using the BCA assay, as it demonstrated the best response with Cel7A and GH7 proteins, which are commonly found in cellulases. Other dye-based assays, like Lowry and Bradford, are less effective at detecting Cel7A.[41] For instance, the reported protein content of hemicellulase (NS22244) has varied widely, with values of 72.80 and 102 mg mL^{-1} estimated using the Bradford and Lowry assays, respectively.[20,42] In this study, the BCA assay measured a protein concentration of 232.02 ± 3.16 mg mL^{-1} for hemicellulase (NS22244), which shows close agreement with previously reported studies (266 mg mL^{-1}).[43] The protein concentration of cellulase (NS22257) was reported as 194 mg mL^{-1} using the Lowry method.[42,44] In contrast, the BCA assay measured it as 379.57 ± 18.83 mg mL^{-1} in this study.

A few studies reported enzyme dosage in terms of volume, in addition to their protein concentration, especially for NS22244 (pre-commercial hemicellulase) and Cellic Ctec3 (an advanced commercial cellulase). 50 μL of hemicellulase (NS22244) and 100 μL of cellulase (Cellic Ctec3) were used per gram of various lignocellulosic substrates that had been pretreated *via* hydrothermal, dilute acid, and alkali processes.[45,46] These volumes are equivalent to 100 L of cellulase and 50 L of hemicellulase when scaled to one ton of lignocellulosic biomass. In a recent study, 200 μL of cellulase (NS22257) and 34 μL of hemicellulase (NS22244) were used per gram of ammonia-pretreated biomass.[35] In this study, 20.83 μL and 6.25 μL of pre-commercial cellulase (NS22257) and hemicellulase (NS22244) were required per gram of biomass, equivalent to 20.83 and 6.25 L per ton of biomass. Compared to earlier studies, cellulase (NS22257) and hemicellulase (NS22244) were significantly reduced to 5- to 10-fold and 5- to 8-fold, respectively.

Previous studies used 88 L of cellulase (20 mg of cellulase protein per g of cellulose) per DT of corn stover to produce 195.32 L of ethanol.[47] The cost contribution of the enzyme accounted for $0.38 per L and was estimated to be reduced by $0.29 per L and $0.22 per L, respectively, by improving the ethanol yields to 254.11 L and 336.52 L. Techno-economics were investigated by further decreasing the enzyme loading (44 L) to 10 mg of cellulase protein per g of cellulose, which could lower the cost of enzyme contribution by $0.19 per L, $0.14 per L, and $0.11 per L for ethanol yields of 195.32 L, 254.11 L, and 336.52 L DT^{-1}, respectively.[14] In the present study, with ED-1, 213.38 L of ethanol is achievable per DT of SS using 20.83 L of cellulase (NS22257) and 6.25 L of hemicellulase (NS22244). Reducing the enzyme dose from 88 L to 27.08 L, with 213.38 L of ethanol DT, can significantly improve the overall process economics of a lignocellulosic biorefinery. With 41.66 L cellulase and 12.5 L hemicellulase (ED-2), 250.63 to 305.21 L of ethanol can be produced per DT of hydrothermal pretreated bioenergy feedstocks.

4 Conclusion

This study demonstrated a clear relationship between sugar concentrations, enzyme dosage, and ethanol yield per ton of biomass. All enzyme dosages (ED-1, ED-2, and ED-3) enabled sufficient sugar release to achieve industrially relevant ethanol titers. However, the increase in sugar yield from ED-2 to ED-3 was marginal (1.98–9.02 g L^{-1}), indicating that ED-2 was the optimal dosage for the tested feedstocks. Economically, ED-1 also holds promise for lignocellulosic biorefineries, as evidenced by ethanol titers of 42.09 ± 2.38 g L^{-1} obtained from sweet sorghum. For an industrial scale, ED-2 (41.66 L of cellulase and 12.5 L of hemicellulase) has the potential to yield 291.11 L of ethanol per dry ton of biomass. In comparison, the same enzyme volume applied at the ED-1 dosage (20.83 L of cellulase and 6.25 L of hemicellulase per ton) will result in 426.76 L across two tons of biomass. With further technological advancements and reductions in enzyme production costs, ED-2 offers the most balanced option for achieving maximum ethanol yield per ton while maintaining economic feasibility.

Author contributions

Narendra Naik Deshavath: conceptualization, methodology, investigation, data curation, and writing – original draft. Mounika Durga Nenavath: investigation. Vijay Singh: funding acquisition, data validation, supervision, and writing – review & editing.

Conflicts of interest

The authors declare that they have no competing financial or personal interests.

Data availability

The data generated for this article are provided in the manuscript. Raw data for figures and tables will be available at CABBI (https://cabbi.bio/datasets/) and in the Illinois Data Bank (https://databank.illinois.edu/).

Acknowledgements

This work was funded by the DOE Center for Advanced Bioenergy and Bioproducts Innovation, U.S. Department of Energy, Office of Science, Biological and Environmental Research Program under Award Number DE-SC0018420. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the U.S. Department of Energy. The authors thank Novozymes North America, USA, for providing the cellulase (NS22257) and hemicellulase (NS22244). The authors thank the Center for Advanced Bioenergy and Bioproducts Innovation for providing the bioenergy feedstocks for this research. The authors also thank Kristen K. Eilts for assisting with the HPLC analysis.

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