Breaking Down Biomass: How Pretreatment and Enzyme Strategy Shape Efficient Bioethanol Yields

Ananda Nanjundaswamy [a,*] and Benedict C. Okeke [b]

Trichoderma sp. SG2, isolated from the Black Belt soils of Alabama, USA, is a potent natural producer of β-glucosidase and a broad spectrum of cellulolytic and xylanolytic enzymes. This study explored the saccharification of lignocellulosic biomass using crude enzymes from *Trichoderma* sp. SG2, various pretreatment strategies, mixed feedstock approaches to enhance sugar yield, and enzyme supplementation to reduce costs. Among the pretreatment methods tested for switchgrass, the most effective was sequential H_3PO_4–ethanol, followed by NaOH–H_2SO_4, H_3PO_4–acetone, H_2SO_4–NaOH, and single-agent treatments (H_2SO_4 alone or NaOH alone). Sugar yields were significantly improved by combining pretreated switchgrass with paper powder as a mixed feedstock. The highest glucose (15.8 g/L) and xylose (3.8 g/L) yields were achieved at 10% pretreated switchgrass after 72 h. A key finding was the significant cost reduction and enhanced saccharification efficiency achieved by supplementing SG2 crude enzyme with 50% of the recommended commercial enzyme dosage. Acid-pretreated switchgrass hydrolysis with SG2 enzyme and commercial enzyme supplementation emerged as the most effective strategy. These results highlight *Trichoderma* sp. SG2 as a promising candidate for developing cost-effective enzyme cocktails for lignocellulosic biomass hydrolysis where 30 to 40% cost of ethanol production process is accounted for enzyme cost.

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INTRODUCTION

Fossil fuels dominate the global energy supply (Liu *et al.* 2018; Popp *et al.* 2021). However, they are non-renewable resources derived from crude oil and coal, and their depletion is imminent (Octave and Thomas 2009; Deswal *et al.* 2011). Additionally, their extraction and combustion contribute to environmental pollution, negatively impacting human health, soil, aquatic life, and climate due to CO_2 emissions and particulate matter (Amjith and Bavanish 2022). The low cost of fossil fuels, along with their adverse environmental effects, presents significant challenges (Ruffell 2008). Furthermore, political instability in oil-producing regions can disrupt supply and drive-up crude oil prices. Rapid industrialization worldwide is further increasing demand, exacerbating these issues. Consequently, extensive research is being conducted to develop renewable, cleaner, and more sustainable alternatives (Wu *et al.* 2006).

Bioethanol production from corn and sugarcane is well-established; however, these feedstocks compete with food and feed applications. In contrast, lignocellulosic biomass, derived from non-food plant material, is the most abundant renewable natural resource (Sánchez and Cardona 2008; Sukumaran *et al.* 2021). The US dry biomass supply is estimated at approximately 1.3 billion tons per year (Popp *et al.* 2021; Langholtz 2024) and is continually replenished through photosynthesis (Ragauskas *et al.* 2006). This natural carbon cycle helps mitigate global carbon emissions. Lignocellulose, a major component of plant biomass, consists of cellulose, hemicellulose, and lignin, with typical compositions of 40 to 50%, 25 to 30%, and 15 to 20%, respectively (Bhardwaj *et al.* 2019; Prasad *et al.* 2019). The vast availability of lignocellulosic biomass makes it a promising raw material for bioenergy production, with the potential to meet rising global energy demands (Danso *et al.* 2022). Converting 1.3 billion tons of cellulosic biomass into biofuel could replace approximately 65% of current fossil fuel usage in the US (Langholtz 2024).

Due to its complex structure, lignocellulosic biomass requires a suite of enzymes to break it down into fermentable sugars. The three main enzyme groups involved are cellulases, xylanases, and ligninases (Sethupathy *et al.* 2021). Cellulose, the most abundant polysaccharide in lignocellulose, is hydrolyzed by endoglucanases (EC 3.2.1.4), exoglucanases (cellobiohydrolases, EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Van Dyk and Pletschke 2012). Xylan, the primary hemicellulose, is degraded by enzymes, such as endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), and acetylxylan esterase (EC 3.1.1.72) (Sunna and Antranikian 1997; Whitaker *et al.* 2003; Ryabova *et al.* 2009). However, the crystalline nature of cellulose and the presence of lignin contribute to the recalcitrance of lignocellulosic biomass, hindering biodegradation. Lignin, a polymer composed of phenylpropane units, further complicates enzymatic hydrolysis (Sangkharak *et al.* 2011). Lignin degradation is facilitated by laccases and various peroxidases, along with auxiliary enzymes such as feruloyl esterase and aryl-alcohol oxidase (Kumar and Chandra 2020). Due to its structural complexity, lignocellulosic biomass requires pretreatment to enhance cellulose and hemicellulose accessibility for enzymatic conversion (Rai *et al.* 2019).

Lignocellulolytic enzymes are produced by various microorganisms, including bacteria (Schwarz 2001; Kim *et al.* 2009; Saratale *et al.* 2010), rumen microbiota (Gharechahi *et al.* 2023), and symbiotic bacteria in termites (Ali *et al.* 2023). However, cellulolytic and xylanolytic fungi, particularly *Trichoderma* sp., remain the primary industrial sources of these enzymes (Saini et al. 2022; de Vries *et al.* 2000; Seyis and Aksoz 2003; Wen *et al.* 2005; Li *et al.* 2010; Wang *et al.* 2018). Among them, the mutant strain *Trichoderma reesei* RUT-C30 is widely used for industrial-scale enzyme production (Seidl *et al.* 2008; Wilson 2009).

Commercial success of cellulosic biofuel production depends on having an efficient saccharification step, which solely relies on biomass hydrolyzing enzymes cellulase and beta-glucosidase. These enzymes at time account for more than 20 to 50% of the total process cost in cellulosic biofuel production (Cherry and Fidantsef 2003; Fang *et al.* 2009; Brijwani *et al.* 2010; Klein-Marcuschamer *et al.* 2012; Johnson 2016). As extensively reviewed by Johnson (2016), the cost of commercial enzymes per gallon of ethanol is estimated to be around \$0.1 to\$ 0.4 per gallon. However, the actual cost of commercial enzyme may vary significantly from \$0.68 to \$1.47 per gallon of bioethanol

produced from biomass. In such a scenario, the best way to reduce cost is to use a combination of crude and commercial enzymes without losing the process efficiency.

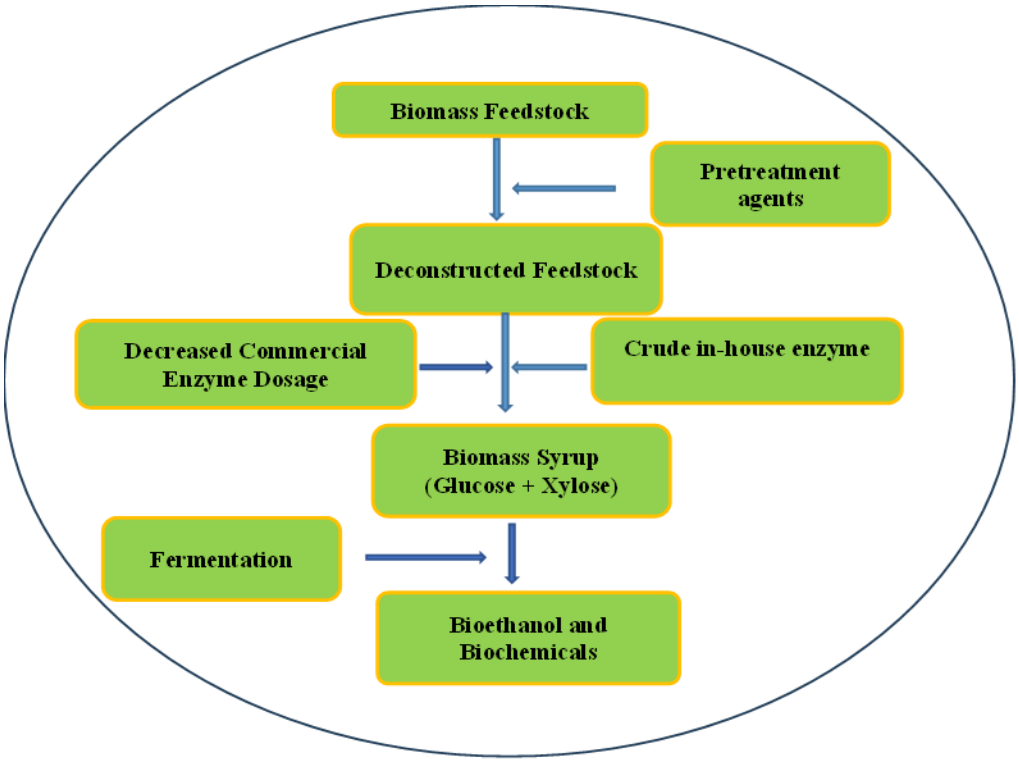

Fig. 1. Overview of bioprocessing system for bioethanol production of crude and commercial enzyme combination

Trichoderma reesei is the most commonly utilized fungus for producing biomass-hydrolyzing enzymes in second-generation biofuel production (Pant *et al.* 2022). However, commercial preparations of *T. reesei* RUT-C30 exhibit low β-glucosidase activity. In contrast, *Trichoderma* sp. SG2, a high β-glucosidase-producing strain isolated from soil-biomass mixtures (Nanjundaswamy and Okeke 2014, 2020a), shows promising enzymatic capabilities. This study evaluated switchgrass biomass hydrolysis using in-house crude enzymes from *Trichoderma* sp. SG2, both independently and in combination with commercial biomass-hydrolyzing enzymes. Crude and commercial enzyme cocktail for saccharification by the novel strain SG2 was the prime focus of this study. An overview of biofuel production is provided in Fig. 1.

MATERIALS AND METHODS

Enzyme Production Process

Seed inoculum was prepared by transferring a loopful of *Trichoderma* sp. SG2 into a 250-mL flask containing 50 mL of sterile high-solids medium, composed of 5% all-purpose flour, 0.1% yeast extract, and 0.1% peptone. The flasks were incubated at 30 °C for 72 h with shaking at 200 rpm.

Crude enzyme production was conducted in 250-mL flasks containing 50 mL of an optimized screening medium with the following components (g/L): 6.2 g powdered waste paper, 9.6 g pulverized switchgrass, 1.4 g peptone, 0.6 g yeast extract, 0.5 g Tween 80 (polysorbate 80), 2 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.003 g $FeSO_4 \cdot 7H_2O$, and 2 mL of Fotch mineral element solution (Focht 1994). The medium was sterilized at 121 °C under pressure for 20 min, cooled to room temperature, and inoculated with approximately 2% of the 72-h-old seed culture per 50 mL of sterile medium. The flasks were then incubated at 30 °C for 5 days with orbital shaking at 200 rpm.

Enzyme recovery and assays for cellulase, xylanase, β-glucosidase, and β-xylosidase activities were conducted as previously described (Okeke *et al.* 2015; Nanjundaswamy and Okeke 2020a). Any variations in the enzyme production process are noted where applicable.

Biomass Pretreatment

The Alamo variety of switchgrass (*Panicum virgatum*) was used. At least eight different pretreatment strategies were compared:

1. 0.5% H_2SO_4 (henceforth referred to as "acid") only
2. 0.5% Acid followed by 0.5% acid
3. 2% Acid only
4. 2% Alkali only
5. 2% Acid followed by 2% alkali
6. 2% Alkali followed by 2% acid
7. 85% Acid followed by acetone
8. 85% Acid followed by ethanol

For pretreatments involving 0.5% or 2% acid and alkali, sulfuric acid and sodium hydroxide were used, respectively. Pretreatment was conducted by soaking approximately 10% (w/v) biomass in the respective acid or alkali solution and autoclaving at 121 °C for 30 min with slow exhaust. After autoclaving, the contents were allowed to cool to room temperature, and the liquid was drained using cheesecloth. The pretreated switchgrass was washed several times with water to remove residual chemicals completely. After 4 to 5 washes, the pH of the liquid was tested and further washed if the pH remained below 5. The washed switchgrass was air-dried at room temperature for 72 h and stored in airtight Ziplock bags until further use.

For sequential acid-alkali pretreatment, switchgrass (10% w/v) was first treated with 2% sulfuric acid and autoclaved at 121 °C for 30 min. The samples were then washed thoroughly with running water until the pH reached 5 to 6. Next, the samples were soaked in 2% sodium hydroxide and autoclaved under the same conditions. After autoclaving, the samples were washed thoroughly until the pH stabilized between 5 and 6, followed by air drying at room temperature for 72 h. A similar sequential method was used for alkali-acid pretreatment.

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

For phosphoric acid pretreatment, modifications to the methods described by Zhang *et al.* (2007) and Sathitsuksanoh *et al.* (2012) were followed. Briefly, 13.125 g of switchgrass was mixed with 100 mL of 85% phosphoric acid and maintained at 50 °C in

a water bath at atmospheric pressure for 60 min. The reaction was terminated by adding 250 mL of 95% ethanol. The samples were centrifuged at 4,500 rpm for 10 min at room temperature. The pellet was resuspended in 250 mL of 95% ethanol, followed by another centrifugation at 4500 rpm for 10 min. The pellet was then washed 3 to 4 times with deionized water until the pH reached 5 to 6. The same method was applied using 85% phosphoric acid followed by pure acetone instead of ethanol.

Compositional Analysis of Biomass

The moisture content of untreated and pretreated biomass was determined using a Mettler Toledo Moisture Meter (HB 43-S Halogen). Approximately 1 g of each sample (pretreated and control) was placed in an aluminum pan, and moisture content was recorded as a percentage using the instrument's preloaded method. At least two readings were recorded per sample.

Duplicate samples were analyzed for the following:

- **Crude fiber:** (Thiex 2009)
- **Acid-detergent fiber (ADF):** (Möller *et al.* 2009)
- **Neutral-detergent fiber (NDF):** (Holst 1973)
- **Cellulose:** (Möller *et al.* 2009)
- **Hemicellulose:** Calculated as NDF – ADF
- **Lignin:** (Van Soest and Wine 1968)
- **Protein:** (Miller *et al.* 2007)
- **Crude protein:** Calculated using the formula: 6.25 × nitrogen value
- **Ash:** (Thiex *et al.* 2012)

Compositional analyses were performed at the Agricultural Experimental Station Chemical Laboratories, University of Missouri, Columbia, MO, USA.

Saccharification

Saccharification was performed in 100-mL flasks with airtight caps. Each flask contained 10 mL of crude enzyme extract and 1 g biomass material. All experiments were conducted in duplicate. Saccharification was carried out at 50 °C with continuous mixing at 80 rpm using a Thermo Scientific MaxQ 4000 shaker. Flasks were tightly sealed to prevent moisture loss. Samples were analyzed for sugar content using HPLC (Ananda *et al.* 2011).

Where specified, enzyme extracted from solid substrate culture was used. *Trichoderma* SG-2 cellulolytic and xylanolytic enzymes were produced *via* solid-state fermentation of soy hulls (Nanjundaswamy and Okeke 2020a). Enzymes were extracted using deionized water containing 2 ppm Lactrol as an antibacterial agent. The extracted enzymes were directly added to the substrate in airtight flasks. For saccharification, 10 g of pretreated material was mixed with 90 mL of enzyme extract. Flasks were tightly sealed to prevent moisture loss and incubated at 50 °C as described above.

Duplicate flasks were maintained for each enzyme treatment and pretreated material. Approximately 100 µL of sample was collected daily for up to 96 h. Samples were diluted, centrifuged, and filtered (0.4 µm) before sugar analysis using HPLC.

HPLC Estimation of Sugars and Ethanol

Sugars and ethanol were quantified using the method outlined in (Ananda *et al.* 2011). Briefly, 100 µL of the supernatant was diluted 1:10 with water, filtered through a 0.45-µm syringe filter, and analyzed using a Shimadzu SIL-20AC HPLC equipped with a refractive index detector (RID-20A,120V) and a column oven (CTO-20A) at 82 °C. Water was used as the mobile phase at a flow rate of 0.6 mL/min. Sugars and ethanol were quantified using a Rezex organic acid column coated with 8% Ca^{++}. Data were acquired using LabSolutions Lite Software. Calibration curves for glucose, xylose, and ethanol were prepared using pure analytical standards obtained from Sigma. Calibration curve concentrations ranged from 1 to 10 mg/mL for sugars and ethanol.

Effect of Feedstock Concentration on Saccharification by Crude *Trichoderma* SG-2 Enzyme

Acid-pretreated switchgrass paper powder was used at concentrations of 2%, 5%, 10%, 15%, and 20%. The experiment was conducted as described under saccharification.

Saccharification of Paper Powder and Supplementation to Switchgrass Feedstock

Office waste (white, printed) shredded paper was milled and stored in Ziplock bags. Acid pretreatment was carried out as described above. Steam pretreatment was conducted using a Sanyo vertical autoclave for 60 min at 121 °C.

In this experiment, virgin (untreated) paper powder at 2% and 5%, as well as steam-pretreated and acid-pretreated samples, were used for saccharification. Enzyme production, saccharification, and sugar estimation were conducted as described above.

Feedstock concentrations of 2%, 4%, 5%, and 6% were tested using a 50:50 mixture of acid-pretreated switchgrass and acid-pretreated paper powder. Enzyme production, saccharification, and sugar estimation were performed as described above.

Sequential Enzyme Addition to Saccharified Switchgrass for Maximum Sugar Yield

SG-2 crude enzyme was produced as described above. Acid-pretreated switchgrass was used at 2% and 5% concentrations. Experiments were conducted in duplicate.

After initial enzymatic treatment for 24 h, samples were centrifuged at 14,000 × g for 15 min using a Beckman Coulter centrifuge. The supernatant was collected for sugar analysis, and fresh crude enzyme was added to the saccharified residue. The mixture was transferred to a flask and allowed to saccharify for another 24 h. This sequential extraction process was conducted to collect data at 24, 48, and 72 h. Enzyme production, saccharification, and sugar estimation were performed as described above.

Biomass Saccharification with SG-2 In-house Enzyme in Conjunction with Commercial Enzymes

A comparative study was conducted on switchgrass saccharification and fermentation using crude and commercial enzymes. Both acid-pretreated and alkali-pretreated switchgrass were tested.

Saccharification was performed in 100-mL flasks with airtight caps, each containing 10 mL of reaction mixture and 2% switchgrass (virgin, autoclaved, acid-pretreated, or alkali-pretreated). All experiments were performed in duplicate.

The following commercial enzymes from Novozymes were used:

- Cellulase (NS22086)
- Xylanase (NS22083)
- β-Glucosidase (NS22118)
- Hemicellulase (NS22002)

Enzyme dosing was adjusted according to Novozymes' recommendations for 2 to 5% (w/v) biomass suspension:

- 5% cellulase
- 0.25% xylanase
- 0.6% β-glucosidase
- 2% hemicellulase

The following enzyme treatments were applied:

1. **C** – Crude aqueous extract of SG-2
2. **N** – Novozymes enzymes at the recommended dosage
3. **C50N** – SG-2 crude extract with 50% of the recommended Novozymes dosage
4. **C25N** – SG-2 crude extract with 25% of the recommended Novozymes dosage

Saccharification was performed at 50 °C with continuous mixing at 80 rpm using a Thermo Scientific MaxQ 4000 shaker. Flasks were tightly sealed to prevent moisture loss. Samples (0.5 mL) were collected at 24-h intervals for up to 72 h and analyzed for sugar content using HPLC (Ananda *et al.* 2011).

Statistical Analyses

Statistical analyses were conducted using SAS (version 9.1.4). Analysis of variance (ANOVA) was performed to compare treatments using 'Procedure (PROC)' Analysis of Variance (ANOVA). Pairwise comparisons were conducted using Tukey's adjustment, with statistical significance set at $P = 0.05$.

RESULTS AND DISCUSSION

Cellulolytic and Xylanolytic Enzymes for Biomass Saccharification

The representative activities of in-house *Trichoderma* SG2 crude enzymes and commercial enzymes are presented in Table 1S. As expected, the levels of cellulolytic and xylanolytic enzyme activities were higher in the concentrated commercial enzymes compared to the unconcentrated crude in-house *Trichoderma* SG2 enzymes. The commercial enzyme also had a higher crude protein content than the in-house enzyme. Consequently, the crude specific enzyme activity of the *Trichoderma* SG2 enzyme was higher compared to that of the commercial enzyme, which exhibited lower specific activity due to its high protein content. The high crude protein content and low specific activity of the commercial enzyme suggest that it underwent multiple rounds of concentration post-fermentation (Hamdan and Jasim 2018).

The unconcentrated crude *Trichoderma* SG2 cellulolytic and xylanolytic enzyme preparation demonstrated significant activity, which could be enhanced through downstream processing methods, such as ultrafiltration or freeze-drying, to obtain a

concentrated enzyme mixture. This would improve biomass saccharification efficiency with *Trichoderma* SG2.

The cellulolytic and xylanolytic enzyme activities of *Trichoderma* SG2 were several orders of magnitude higher when produced in solid-substrate fermentation (Nanjundaswamy and Okeke 2020a). Additionally, dilution of the enzyme prior to assay revealed significantly higher activity—approximately 2.8 times for cellulase and 7.9 times for xylanase—in the enzymes obtained from liquid-submerged culture fermentation (Okeke *et al.* 2015).

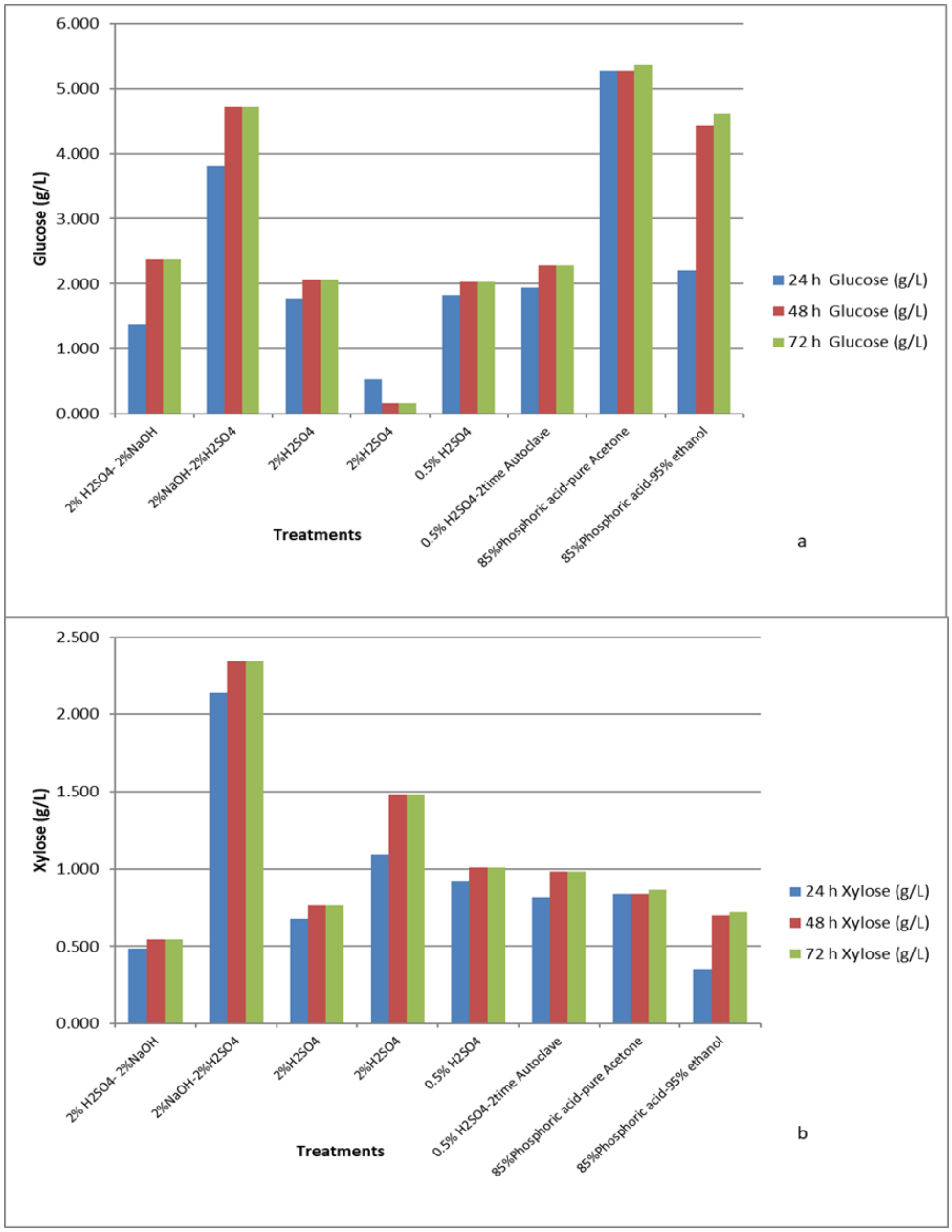

Fig. 2. Effect of different pretreatment methods on saccharification of switchgrass (Standard error less than 0.5%)

Effect of Pretreatment Methods on Biomass Saccharification

Composition of switchgrass used for pretreatment is provided in Table 2S. In the present investigation the authors used switchgrass from Union Springs. The composition profile of switchgrass used in the present investigation was similar to the commercially produced switchgrass with respect to cellulose, hemicellulose, lignin, protein, and ash content (Tumuluru 2015; Wang *et al.* 2020; Wasonga *et al.* 2025). Different biomass pretreatment methods were evaluated. Figures 2a and 2b present an initial investigation into the saccharification of sodium hydroxide-pretreated switchgrass using 5%, 10%, and 20% dilutions of crude *Trichoderma* SG2 enzyme treatments over 48 and 72 h. Glucose release from sodium hydroxide-pretreated switchgrass was approximately 0.35 g/L with 10% and 20% crude *Trichoderma* SG2 enzyme loading. Increasing the incubation time from 48 to 72 h did not significantly affect glucose release (Fig. 1a). A similarly low sugar yield was observed for xylose release, which was approximately 0.14 g/L at 10% and 20% enzyme loading. The duration of substrate-enzyme incubation had little impact on sugar yield, except for a moderate increase at 5% crude *Trichoderma* SG2 enzyme loading (Fig. 1b).

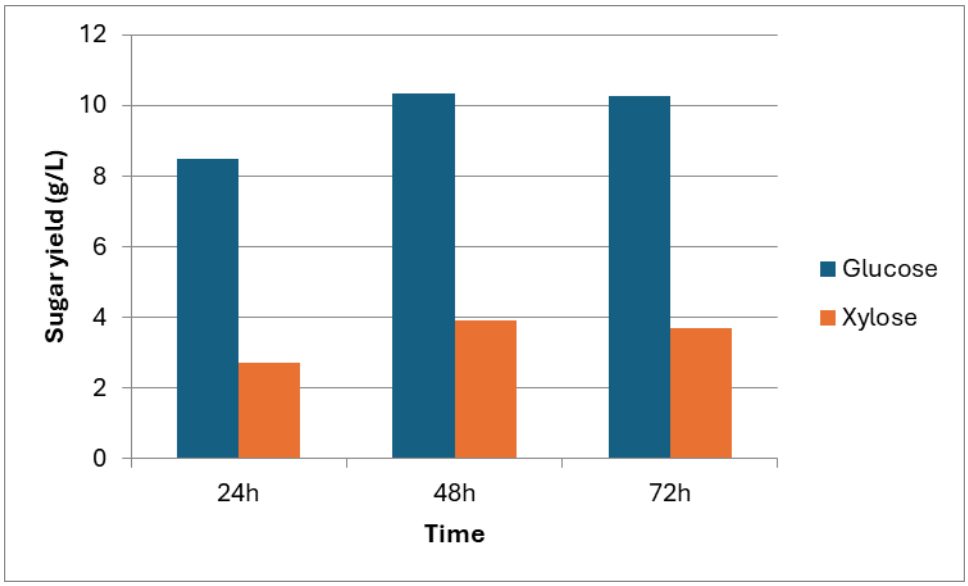

Fig. 3. Saccharification of acid-pretreated paper powder (Standard error less than 0.5%)

The low glucose and xylose yields from alkali pretreatment may be attributed to the low concentration of crude SG2 enzymes at 5% to 20% loading. Sodium hydroxide has been used for biomass pretreatment in concentrations ranging from 0.5% to 10%, heated up to 180 °C for 60 min (Kim *et al.* 2016). Optimal NaOH pretreatment was achieved using 3% NaOH with heating in a laboratory autoclave for 60 min (Sindhu *et al.* 2015). Saccharification of wheat straw pretreated with sodium hydroxide was optimal at 1.0% (w/w) NaOH, 5.0% (w/v) substrate at 121 °C for 60 min, using cellulase from *Trichoderma reesei* ATCC 26921 (Wang *et al.* 2021). Furthermore, glucose and xylose yields do not account for all saccharide products, such as oligosaccharides such as cellobiose and other cello-oligosaccharides, as well as xylobiose and other xylo-oligosaccharides, which were not targeted in the HPLC analysis. The mechanism of NaOH pretreatment involves the solubilization of hemicellulose and lignin by breaking

ether and ester bonds linking carbohydrates to lignin, as well as C-C and ester bonds in lignin (Kim *et al.* 2016).

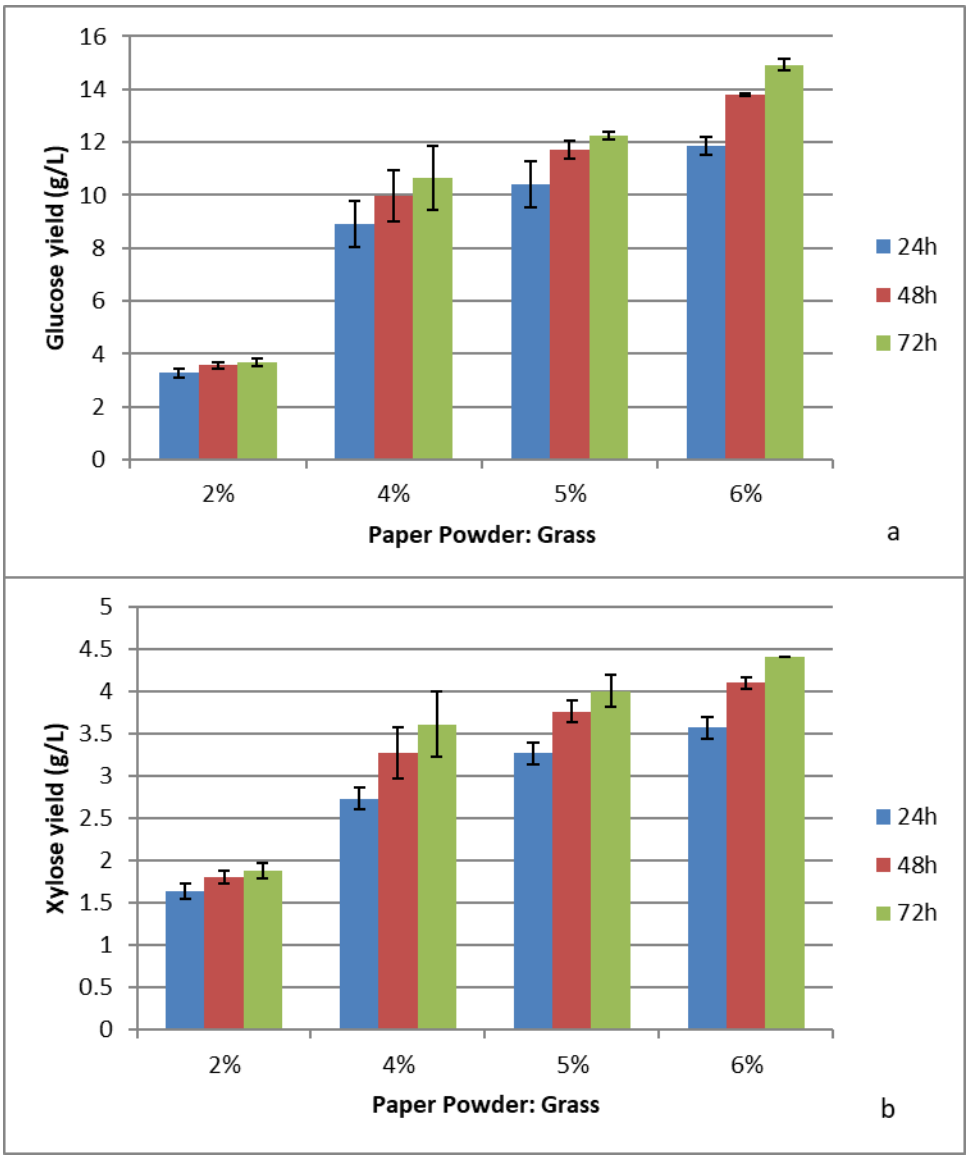

Fig. 4. Paper powder supplementation of switchgrass: a. glucose, b. xylose; means and standard errors are indicated (paper powder:switchgrass = 1:1)

Up to 10 times the sugar yield from alkali pretreatment was observed with acid pretreatment of switchgrass. The highest glucose release after 72 h of incubation was 3.6 g/L at 20% enzyme loading (Fig. 2a). Sugar yield was directly related to reaction time and enzyme loading. A similar trend was observed for the release of xylose from acid-pretreated switchgrass (Fig. 2b). At 20% enzyme loading and 72 h of saccharification, the highest xylose release was 2.2 g/L. To enhance sugar yield from pretreated biomass, enzyme loading was increased to 50% and 100% crude *Trichoderma* SG2 enzyme. With 2% sulfuric acid pretreatment, a glucose yield of 9.7 g/L was observed at 72 h, which was not statistically different from the yield recorded after 96 h of saccharification using 100% crude *Trichoderma* SG2 cellulolytic-xylanolytic enzyme complex (Fig. 2a). The

highest xylose yield was 0.36 g/L at 96 h with 100% crude enzyme, which did not vary significantly from the result at 72 h (Fig. 2b). Overall, crude enzyme loading at 100% yielded the highest total glucose yield of approximately 10 g/L and was significantly more effective than 50% crude enzyme loading for saccharification.

Reports suggest that acid pretreatment enhances lignocellulose biomass saccharification by disrupting hydrogen and covalent bonds, as well as Van der Waals forces interlinking macromolecules (cellulose, hemicellulose, and lignin) in lignocellulose (Li *et al.* 2010). Consequently, cellulose becomes more accessible for enzymatic hydrolysis, hemicellulose (especially xylan) is largely dissolved, and lignin persists. Acid pretreatments using H_2SO_4, HCl, and CH_3COOH at concentrations ranging from 1% to 4% have been employed to improve lignocellulose biomass digestibility (Amin *et al.* 2017).

A study on acid-pretreated sugarcane bagasse saccharification using a cellulase cocktail from *Talaromyces verruculosus* IIPC 324 and commercial biofuel enzymes found that the in-house enzyme achieved 59% saccharification, whereas Cellic CTec2 reached 77% (Jain *et al.* 2019). Acid pretreatment typically requires high temperatures, increasing energy input and potentially leading to the formation of inhibitory products, such as furfural and 5-hydroxymethylfurfural (HMF), which result from the dehydration of saccharides such as xylose, galactose, mannose, and glucose (Hendriks and Zeeman 2009).

Overall, dilute sulfuric acid pretreatment followed by saccharification with 100% crude enzyme (unconcentrated culture supernatant) resulted in the most effective saccharification, yielding 10 g/L of sugar from switchgrass. This work supports in-house enzyme production without requiring concentration, allowing biomass to be directly applied to the clarified fermentation broth without further clarification. This reduces equipment and labor costs associated with downstream clarification by centrifugation or filtration.

Supplementation of Switchgrass Feedstock with Paper Powder

The saccharification of acid-pretreated paper powder showed that 5% acid-pretreated paper powder yielded the highest glucose concentration of 10.3 g/L after 48 h, starting with 8.5 g/L at 24 h. The highest xylose concentration was 3.9 g/L at 48 h, starting with 2.7 g/L at 24 h (Fig. 3). Sugar yields increased up to 48 h and then stabilized, showing no significant difference between 48 and 72 h. In un-pretreated and autoclaved paper powder, sugar yields were negligible, with less than 0.5 g/L after 24 h and undetectable levels after 48 h.

The effect of supplementing switchgrass with paper powder in equal proportions (1:1) to achieve total concentrations of 2%, 4%, 5%, and 6% is shown in Figs. 4a and b. At 6% feedstock concentration (paper powder: switchgrass), both glucose and xylose yields significantly increased ($P < 0.0001$) in the following order: 6% > 5% > 4% > 2%. Sugar yields increased significantly ($P < 0.0001$) with reaction time: yields at 72 h > 48 h > 24 h for both glucose and xylose. Analysis of each concentration revealed that glucose and xylose yields did not vary significantly over time for the 2%, 4%, and 5% concentrations. However, yields at 6% showed significant differences, with the highest yields at 72 h (72 h > 48 h > 24 h for glucose and xylose). The highest glucose yield (15 g/L) and xylose yield (4.4 g/L) were obtained from 6% pretreated switchgrass feedstock after 72 h of saccharification. Using a mixed feedstock approach has the potential to

reduce costs compared to using a single feedstock system (Oke *et al.* 2016) and can also reduce potential inhibitors from biomass pretreatment depending on the supplementary feedstock, thereby improving saccharide yields. Mixed-feedstock alkaline treatment enhanced lignin recovery from residual lignocellulosic materials from *Cannabis* and *Euphorbia* species processing for value-added pharmaceutical metabolites (Berchem *et al.* 2020).

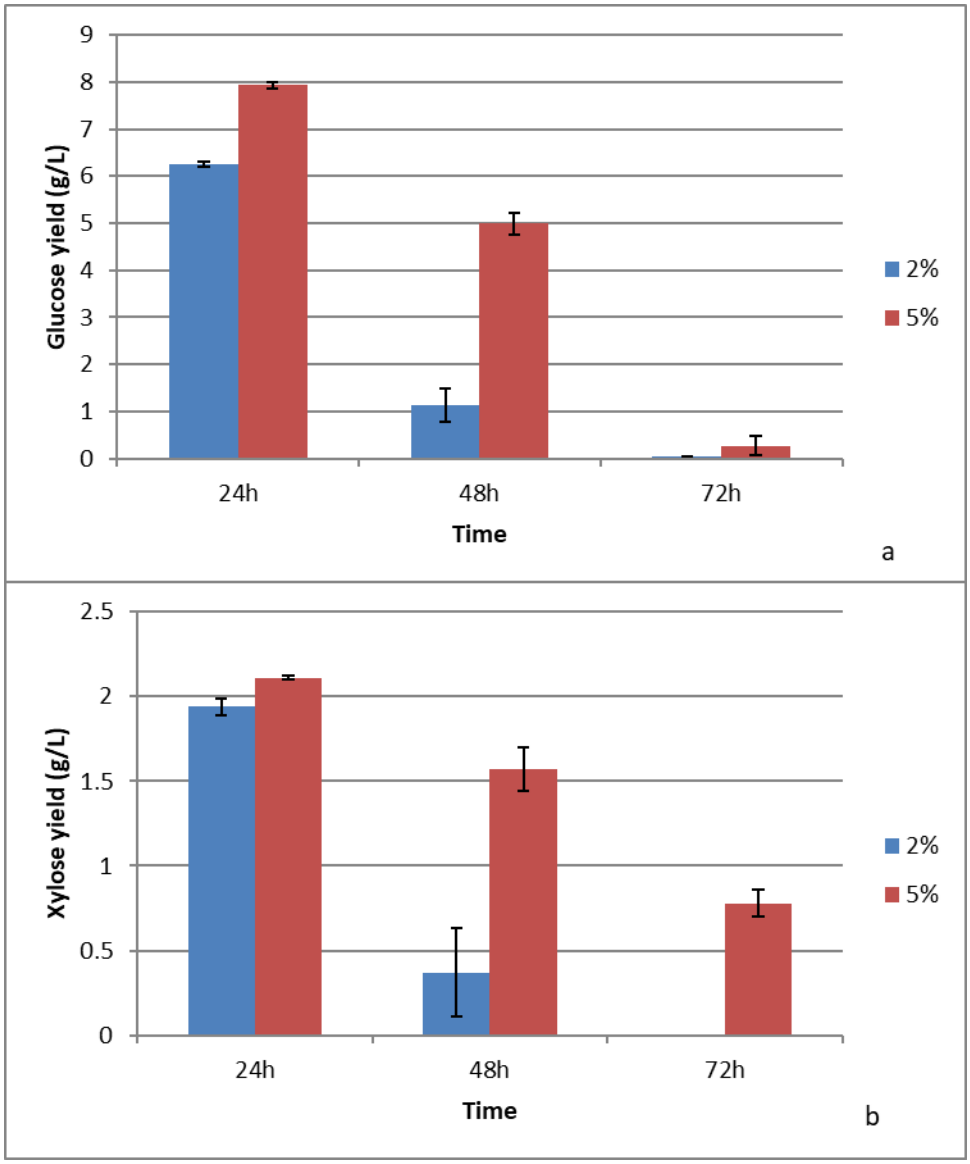

Fig. 5. Sequential saccharification of saccharified-switchgrass feedstock: a. Glucose, b. xylose; means and standard errors are indicated

The great potential of mixed-feedstock processes to produce biofuels and biochemicals was demonstrated using a mixture of corn stover, switchgrass, lodgepole pine, and eucalyptus, yielding a 90% sugar yield within 24 h of saccharification (Shi *et al.* 2013). Saccharification of acid-pretreated waste office paper powder at 5% concentration was most efficient and yielded 10 g/L glucose after 48 h. Consequently, shredded white-printed paper waste can be utilized for saccharification and potential

biofuel and biochemical production. Furthermore, it can be used to supplement switchgrass feedstock. Supplementation of paper powder to switchgrass feedstock at a 50:50 ratio (50% of each to reach the final concentration) showed that a 6% total substrate concentration resulted in the highest glucose and xylose levels (Figs. 3a, b), slightly greater than those obtained with 5% switchgrass alone (Figs. 2a, b).

Effect of Sequential Enzyme Addition to Switchgrass Biomass Saccharification

The potential to enhance saccharide yield through the sequential addition of enzymes was assessed. In the initial addition of enzyme, 6.26 g/L and 5 g/L of glucose and 1.9 g/L and 1.6 g/L of xylose were observed from saccharification of 2% and 5% acid-pretreated switchgrass, respectively, after 24 h of saccharification (Figs. 5a and b). A subsequent addition of SG2 crude enzyme to the same biomass (second saccharification) produced 1.4 g/L and 5 g/L of glucose and 0.4 g/L and 1.6 g/L of xylose, respectively, from 2% and 5% acid-pretreated switchgrass. The third saccharification resulted in 0.05 g/L and 0.3 g/L of glucose and 0 g/L and 0.8 g/L of xylose, respectively, from 2% and 5% switchgrass. After repeated enzyme addition and saccharide extraction over 72 h, a cumulative total of 7.5 g/L and 10.3 g/L of glucose and 2.3 g/L and 3.9 g/L of xylose were achieved from 2% and 5% switchgrass biomass saccharification, respectively. The lower xylose yield is not unexpected, because dilute acid pretreatment significantly dissolves hemicellulose.

Optimizing the process of repeated saccharification of saccharified switchgrass (intermittent dosing) can enhance saccharification and subsequent conversion to ethanol and biochemicals. In a similar study, a pre-hydrolysis step in a simultaneous saccharification and fermentation (SSF) process increased ethanol production (Tareen *et al.* 2021). The authors also showed that a fed-batch process at 10% and 20% biomass decreased high viscosity issues, improved hydrolysis, and subsequently increased ethanol yield.

Biomass Saccharification with *Trichoderma* SG2 In-House Enzyme in Conjunction with a Commercial Enzyme

The saccharification of acid-pretreated switchgrass was evaluated using *Trichoderma* SG2 in-house enzyme (N) in combination with a commercial enzyme (C). The highest glucose yield was observed with a 50:50 combination (50% C and 50% N) and a 25:75 combination (25% C and 75% N) (Fig. 6a). Glucose yields followed the trend: C50N > C25N > N > C. The highest glucose yield of 5.61 ± 0.1 g/L was obtained with C50N after 48 h, with no significant increase observed at 72 h. The next highest yield of 5.11 ± 0.3 g/L was recorded with C25N after 72 h. Xylose release exhibited a similar pattern (Fig. 6b), with the highest yield of 1.64 ± 0.02 g/L after 72 h using C50N.

Switchgrass pretreated with 2% sulfuric acid and hydrolyzed with 100% crude enzyme at 30 °C resulted in the highest residual glucose (2.7 g/L, Fig. 6a) and xylose (2.4 g/L, Fig. 6b) at 24 h. However, a reduction in sugar content over time was observed in Control 2, suggesting microbial growth, potentially due to *Trichoderma* SG2 from the crude enzyme.

Combining the in-house crude enzyme with commercial enzymes yielded remarkable results and demonstrated potential cost reductions compared to using either enzyme separately for biomass saccharification. While the commercial cellulase and

beta-glucosidase exhibited higher activity than the *Trichoderma* SG2 crude enzymes, the *Trichoderma* SG2 xylanase had twice the activity of the commercial xylanase. Acid-pretreated switchgrass hydrolyzed with SG2 crude enzyme supplemented with 50% of the recommended commercial cellulase dosage (C50N) achieved the highest sugar yield after 72 h. Using *Trichoderma* SG2 crude enzyme supplemented with Novozymes cellulase at 25% or 50% of the recommended dosage (C25N or C50N) increased glucose yield 19% to 30% compared to commercial enzymes alone.

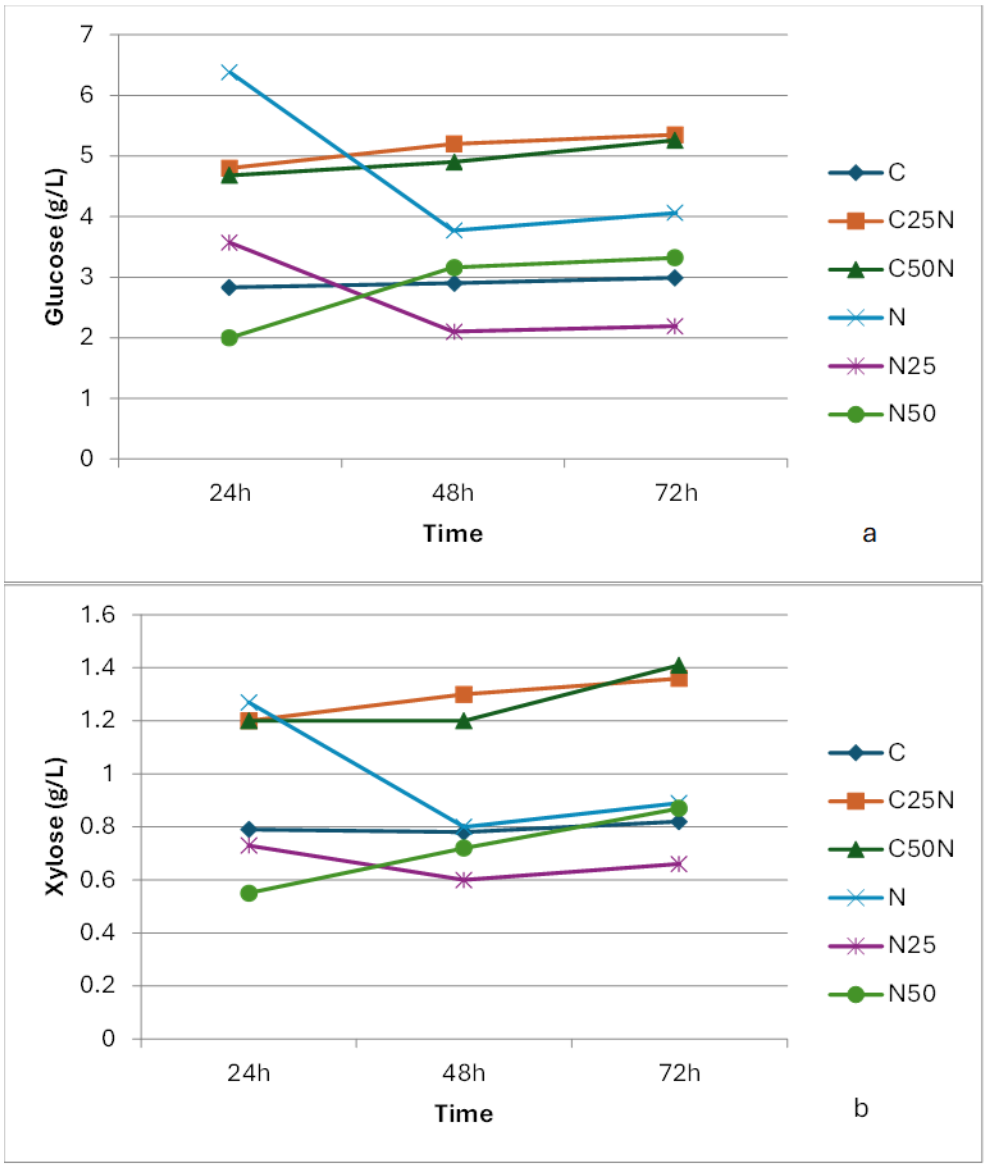

Fig. 6. Switchgrass saccharification by *Trichoderma* SG2 and commercial enzyme (N)

Supplementing commercial enzymes with 50% SG2 crude enzyme offers a cost-effective alternative by reducing commercial enzyme usage while improving efficiency, achieving at least 30% higher glucose yield. Because *Trichoderma* SG2 crude enzyme is derived from fermentation broth without expensive chemicals or complex processes, its production is economically feasible for in-house use at farms or biorefineries.

Fermentability studies of biomass hydrolysate using this enzyme mix showed a maximum fermentation efficiency of 74% with C50N, followed by 65% with C25N, 55% with N, and 52% with C. Encouraging results were also observed with 25% commercial enzyme supplementation of crude enzyme, potentially reducing enzyme costs by up to 75%. Even a 25% reduction in commercial enzyme costs is significant, given that enzymes are a major expense in cellulosic ethanol production.

A combination of crude enzyme and 50% commercial enzyme yielded 5.61 g of glucose from 20 g of acid-pretreated switchgrass at 48 h, indicating a potential yield of 56.1 g of glucose from 200 g of biomass. Similarly, this combination produced 1.64 g of xylose from 20 g of biomass, translating to a potential yield of 16.4 g of xylose from 200 g. Thus, acid-pretreated switchgrass has the potential to yield a total of 72.5 g of sugars (glucose and xylose) from 200 g of biomass.

Optimizing enzyme cocktail composition is crucial for enhancing hydrolysis efficiency in lignocellulosic biomass saccharification (Zhang *et al.* 2023). *Trichoderma* SG2 produces a promising mix of cellulolytic and xylanolytic enzymes (Nanjundaswamy and Okeke 2020b). In contrast, *Trichoderma* RUT-C30, a widely used industrial strain, produces lower beta-glucosidase levels, necessitating the development of advanced biomass hydrolyzing enzymes such as Celluclast, Cellic CTec2, and Cellic CTec3 to optimize enzyme balance and enhance hydrolysis.

CONCLUSIONS

1. Cost-effective biomass deconstruction and enzyme production are essential for efficient lignocellulosic bioprocessing.
2. Sequential phosphoric acid-acetone pretreatment yielded the highest sugar release, followed by acid-alkali pretreatment, with the latter being more economical at low concentrations.
3. *Trichoderma* SG2 produces a promising enzyme cocktail for biomass saccharification, enhancing sugar yield when combined with 25 to 50% commercial enzyme dosage, significantly reducing costs.
4. Mixed feedstock saccharification further improved sugar recovery. Future research will focus on optimizing saccharification and fermentation processes and enhancing enzyme production through gene cloning and overexpression for improved biomass hydrolysis.
5. This study offers valuable insight into evaluating enzymatic activity across both crude and commercial enzyme formulations, enabling strategic supplementation of enzyme blends to maximize sugar yields, a key factor in advancing cost-effective cellulosic biofuel production.

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APPENDIX

Supplementary Material

Table S1. Enzyme Activities of 'Commercial and In-house *Trichoderma* SG2 Crude Enzyme

Enzymes	Activity (U/mL/30min)	Protein (mg/mL)	Specific Activity (µ/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

Notes: In-house crude enzyme had higher specific activity indicator of high specificity to the substrate. In this case cellulose and hemicellulose.

Table S2. Compositional Analyses of Feedstock Samples Used in this Study

W/W%	Crude protein	Crude fiber	ADF	NDF	Ash	Cellulose	Lignin	Hemicellulose	Reducing Sugars
Union Springs (old) switchgrass	6.31	33.95	40.52	78.3	4.76	33.89	5.75	37.78	3.07
Acid-treated Union Springs (old) switchgrass	5.3	48.9	64.96	76.06	2.44	53.12	10.15	11.1	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	82.3	84.63	0.52	59.34	22.87	2.33	0.4

Notes: Biochemical composition of switchgrass used in the study. We compared locally grown two switchgrass from Union Springs and USDA ARS Agricultural Station in Auburn.