

Bioethanol Production from Two-Stage Hydrothermally Pretreated Sugarcane Bagasse: Enzymatic Saccharification, Fermentation Optimization and Mass Balance Analysis

Ravi Kumar

ravisangwan11@gmail.com

National Dairy Research Institute

Nitin Kumar

Chaudhary Charan Singh Haryana Agricultural University

Sonu Saini

Anand School of Engineering and Technology, Sharda University,

Charan Singh

Chaudhary Charan Singh Haryana Agricultural University

Gaurav Chaudhary

Chaudhary Charan Singh Haryana Agricultural University

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Abstract

Lignocellulosic biomass valorization for second-generation bioethanol production has gained considerable attention as a sustainable substitute for fossil fuels. Sugarcane bagasse (SCB) is an abundant agro-industrial residue rich in fermentable polysaccharides and represents an ideal feedstock for this purpose. The present study optimized enzymatic saccharification and fermentation of SCB subjected to a two-stage hydrothermal-mechanical pretreatment that achieved 53.01% lignin removal. The optimized process also resulted in a 1.71-fold increase in available glucan content. Cellulolytic and hemicellulolytic enzymes were produced in-house from *Trichoderma viride* and *Trichoderma harzianum* under wheat bran-based solid-state fermentation and subsequently partially purified by ammonium sulfate precipitation. *T. viride* yielded the highest enzyme activities—FPase: 8.09 FPU/g, CMCase: 6.33 IU/g, xylanase: 9,104 IU/g reaching peak productivity on day 6 of incubation. Partial purification improved fold-increase to 8.63 (FPase), 9.11 (CMCase), and 6.45 (xylanase) for *T. viride*, and 10.87 (FPase), 8.59 (CMCase), 7.23 (xylanase) for *T. harzianum*. Enzymatic saccharification of pretreated SCB at 15 FPU/g over 40 h released 417.40 mg/g and 387.32 mg/g total reducing sugars with *T. viride* and *T. harzianum* cellulases, respectively. An artificial enzyme cocktail at 20 FPU/g yielded 423.88 mg/g, corresponding to a saccharification yield of 68.12%. Fermentation of sugar hydrolysates by *Saccharomyces* sp. at 28 °C over 24 h achieved a maximum bioethanol yield of 6.39 g/L (0.43 g/g; 43.17%; 86.34% of theoretical maximum) using *T. viride*-saccharified hydrolysates, confirmed by both spectrophotometric and gas chromatographic analysis. Mass balance estimation projected 3,263 L of bioethanol per hectare of sugarcane cultivation, establishing the industrial scalability of this eco-friendly, chemical-free pretreatment platform.

Highlights

- SCB pretreatment removed 53% lignin and improved glucan by 1.71-fold
- Enzymatic hydrolysis produced 417 mg/g sugars with 68.1% saccharification yield
- produced 6.39 g/L ethanol, reaching 86.3% of theoretical yield
- Mass balance projected ethanol production 3,263 L/ha sugarcane biomass

1. Introduction

The rapid industrialization and modernization have triggered an unusual rush in our demand for energy. Unfortunately, this hunger for energy is mainly encountered by finite and rapidly depleting fossil fuels (Saini et al., 2023). However, the combustion of fossil fuels significantly contributes to greenhouse gas emissions (GHGs) and global warming, resulting in an acceleration of environmental problems such as climate change, ocean acidification, and biodiversity loss (Birania et al., 2022; Romero-Perdomo et al., 2022). In this context, it is necessary to explore sustainable and renewable energy sources. Biofuels, derived from biological materials, especially lignocellulosic biomass (LCB), have emerged as a promising

substitute to meet the ever-growing energy demand. Their renewable nature has the potential to make a substantial impact on reducing GHGs (Baksi et al., 2023; Sher et al., 2021). The burning of biofuels releases the least amount of CO₂, which can be absorbed by plants during their growth, creating a closed carbon cycle. Replacing fossil fuels with novel biofuels has effectively lowered our carbon footprint and slowed down the progression of global warming (Cantarella et al., 2023).

Lignocellulosic biomass refers to a broad range of organic materials primarily made up of cellulose, hemicellulose, and lignin; the key components in plant cell walls responsible for providing structure, strength, and rigidity (Kumar et al., 2024a; Saini et al., 2023). Cellulose and hemicellulose, being polysaccharides can be solubilized to fermentable sugars, while lignin is a complex aromatic polymer that adds hindrance to biomass fractionation (Akram et al., 2024; Suman et al., 2024). Agricultural residues like straws, stubs, and leaves, forestry waste such as wood chips and sawdust, and dedicated energy crops like switchgrass and *Miscanthus*, etc are some important types of LCB (Prasad et al., 2024; Saini et al., 2023). These LCBs are not only cost-effective resources for biofuel production but also do not compete with the food supply, a crucial distinction from first-generation feedstocks.

Sugarcane bagasse (SCB), the fibrous residue of a by-product of the sugar industry, shares the common lignocellulosic structure. It is generated globally in large quantities, especially in countries with significant sugarcane production. India, the world's second-largest sugarcane producer, generates in excess of 100 million tonnes of SCB annually (Kumar et al., 2024a, 2020). The wide availability and high cellulose and hemicellulose establishes SCB as a sustainable and economically feasible resource for biofuels production (Sun et al., 2024). The conversion of SCB into bioethanol involves a complex series of steps that blend biological and engineering principles. Initially, processing methods including chopping and grinding are required to fragment the biomass into smaller particles to increase its surface area (Batista et al., 2019; Suman et al., 2024). These methods also reduce the cellulose crystallinity and decrease the degree of polymerization. Further, a pretreatment method is always necessary to break down the recalcitrance of LCB, especially lignin polymer (Saini et al., 2023; Sunar et al., 2024). Numerous pretreatment strategies have been developed and optimized which includes physical, chemical, physicochemical, and biological, to fractionate the LCB (Birania et al., 2021; Chen et al., 2022; Kumar et al., 2024a). Though every pretreatment method possesses certain challenges (Saini et al., 2024). For instance, physical methods demand significant energy and also have low lignin degradation efficiency. Acid pretreatment can lead to the production of fermentation-inhibitory substances such as furan, furfural, formic acid, and acetic acid (Gopinath et al., 2024). Meanwhile, alkali pretreatment requires high chemical usage and processing cost (Kundu et al., 2023). Biological pretreatment methods are, although environmentally benign, demanding a long processing time to remove the desired lignin content. To address these issues, two-stage pretreatment methods have been explored to maximise lignin degradation with minimum sugar loss. The integration of mechanical and hydrothermal processes in the two-stage pretreatment has been shown to significantly boost the efficiency of enzymatic hydrolysis and bioethanol production (Juneja et al., 2021; Kumar et al., 2024a). After a successful delignification method, the remaining sugar-rich portion can be dissolved into individual fermentable sugars using either acidic or enzymatic routes (Saini et al., 2023). Enzymatic routes are always preferred due to their

ability to produce sugars that are largely devoid of inhibitory compounds. The readily available fermentable sugars are then converted into bioethanol through microbial fermentation, especially by yeast that metabolizes the sugars into ethanol and carbon dioxide (De Oliveira Rodrigues et al., 2022; Kumar et al., 2023).

Over time, numerous studies have been published dealing with various pretreatments, saccharification, and biofuel production from a wide range of LCBs (Chen et al., 2022; Cheng et al., 2022; Gopinath et al., 2024; Prasad et al., 2024). Still, many research questions remained untackled in the field of biofuels production from LCB employing environmentally friendly processes. Therefore, in the current investigation, it is aimed to optimize enzymatic hydrolysis of pretreated SCB using a laboratory-produced crude fungal enzymatic cocktail. Further, the sugar hydrolysates were fermented using a wild *Saccharomyces* sp. for efficient bioethanol production. Finally, the mass balance study of our optimized process will pave the way in developing a vital solution for utilization underutilized LCB for efficient biofuel production employing eco-friendly processes with the aim of a futuristic circular bioeconomy.

2. Materials and methods

2.1. Chemicals and Reagents

All chemicals and reagents used in this study were of analytical-grade, commercially available unless otherwise specified. Specific chemicals, including 3,5-Dinitrosalicylic acid (DNS), carboxymethyl cellulose (CMC), beechwood xylan, and absolute ethanol, were procured from Sigma-Aldrich (St. Louis, MO, US). Culture media components (Potato Dextrose Agar, yeast extract, dextrose, peptone, agar) and sugar analytical standards (glucose, xylose, mannose, phloroglucinol, cellobiose, arabinose, galactose, xylitol, sucrose, sorbitol, and maltose) were obtained from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). All remaining chemicals, buffers, and mineral salts were of the highest commercially available purity grade.

2.2. Feedstocks and Biomass Preparation

The two-stage hydrothermally pretreated SCB biomass was obtained from the Department of Processing and Food Engineering, CCSHAU, Hisar, Haryana, India, following the optimized protocol described by Kumar et al. (2024). Briefly, raw SCB was subjected to hydrothermal pretreatment at 190°C for 11.5 min in stainless steel autoclave reactors (solid: water ratio = 1:3, w/w), followed immediately by three sequential passes of wet disk milling (Quaker City grinding mill model 4E) without intermediate washing or neutralization. The two-stage pretreated biomass, characterized by 53.01% lignin removal and 1.71-fold glucan enrichment relative to raw SCB (glucan: 63.76%, xylan: 13.47%, acid-insoluble lignin: 10.01%), was stored in airtight polyethylene bags at 4°C under dry conditions to prevent moisture uptake, microbial contamination, and compositional change.

Wheat bran was procured locally and used as the sole carbon source for fungal enzyme production. It was washed thoroughly with running tap water to remove residual flour fines. Then the bran was

subjected to drying in a solar tunnel dryer (a low-cost drying method based on solar energy) to reduce the biomass moisture content below 10.0%, (dry weight basis). The processed wheat bran was stored in airtight bags under ambient dry conditions until use. (Kumar et al., 2023; Saini et al., 2022).

2.3. Microorganisms and Culture Maintenance

The fungal cultures, namely *Trichoderma viride* and *Trichoderma harzianum*, utilized in the present investigation were a kind gift from the Department of Plant Pathology, CCSHAU, Hisar, India. Both ascomycete fungal cultures were maintained on Potato Dextrose Agar (PDA) slants at 4 °C and subcultured monthly to maintain viability. The basidiomycetous yeast *Saccharomyces* sp. used for ethanol fermentation was obtained from the Bioenergy Laboratory, Department of Renewable and Bio-Energy Engineering (RBEE), CCSHAU, Hisar, India and maintained on Yeast Extract Peptone Dextrose (YEPDA) slants at 4 °C.

2.4. Enzyme Production and Partial Purification of Cellulases and Xylanases by Solid-State Fermentation

Cellulase (CMCase, FPase) and xylanases were produced under solid-state fermentation (SSF) conditions. Fresh fungal spores of *T. harzianum* and *T. viride* were used as an inoculum (Kumar et al., 2023). Ten grams of wheat bran (washed and sun-dried) were transferred to a 250 mL Erlenmeyer flask and moistened with mineral salt solution (containing, per litre: 2.0 g KH_2PO_4 , 1.4 g $(\text{NH}_4)_2\text{SO}_4$, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g CaCl_2) to achieve 75% moisture content. Flasks were plugged with non-absorbent cotton, autoclaved at 125°C for 20 min and cooled to the ambient temperature. Each flask was uniformly inoculated with freshly prepared fungal spores (1×10^6 /g of dry substrate) and incubated under static conditions for up to 7 days at $30 \pm 1^\circ\text{C}$ (Chutani and Sharma, 2016; Kumar et al., 2023).

After fermentation, crude enzyme extracts were harvested by adding 50 mM sodium acetate buffer (pH 5.0 ± 0.2) at a solid: buffer ratio of 1:10 (w/v), agitating at 150 rpm for 1 h at 4°C, and filtering through four layers of muslin cloth. The filtrates were centrifuged at $9,800 \times g$ for 12 min at 4°C to obtain biomass-free supernatants for enzyme activities.

Enzyme activities of CMCase, FPase, and xylanase were evaluated using a 2.0% (w/v) CMC solution, a strip of Whatman No. 1 filter paper (measuring 1×6 cm, approximately 50 mg), and 0.6% (w/v) beechwood xylan as substrates, respectively. For each assay, 500 µL of appropriately diluted enzyme extract was mixed with 500 µL of substrate in 50 mM sodium acetate buffer (pH 5.0) and incubated at 50°C for 10 min under agitation. The sugars that were subsequently released were quantified using the dinitrosalicylic acid (DNS) method (Miller, 1959). One International Unit (IU) of enzyme activity was defined as the quantity of enzyme that is necessary to liberate 1 µmol of reducing sugar per min per mL under the conditions specified for the assay. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard to enable calculation of specific activity (IU/mg protein).

The cellulases and xylanases were partially purified by adding fractional ammonium sulfate precipitation at 4°C. The enzyme extract was subjected to sequential saturation at 15% and 60% ammonium sulfate. The resulting protein precipitates were collected at 60% saturation and recovered by centrifuging them at 10,000×g for 15 min at 4°C. These precipitates were then resuspended in the smallest possible volume of 50 mM sodium acetate buffer (pH 5.0) and then dialyzed overnight at 4°C against three changes of the same buffer using a 12 kDa cut-off dialysis membrane. Fold increase in specific activity was calculated as the ratio of specific activity after purification to that of the crude extract (Saini et al., 2022).

2.5. Enzymatic Hydrolysis of Pretreated SCB

2.5.1. Enzymatic Hydrolysis of Pretreated SCB Using Hydrolases

Enzymatic hydrolysis of pretreated SCB was carried out using partially purified hydrolases produced from *T. harzianum* and *T. viride*. The enzymatic hydrolysis was performed at 5% (w/v) consistency with 50 mM acetate buffer (pH 5.0) supplemented with 0.5% Tween-80 and 0.005% sodium azide. In brief, a 250 ml Erlenmeyer flask containing 2.5 g of pretreated SCB was mixed with 50 ml of the aforementioned buffer solution, along with 250 µl of Tween-80 and 0.0025 g of sodium azide. Before enzyme addition, the biomass was conditioned by incubating it at 50 °C and 150 rpm for 2 hours in a shaking incubator. The resulting slurry was then supplemented with partially purified enzymes at a dosage ranging from 5 to 20 FPU/g of the substrate, and the reaction was allowed to proceed at 50 °C for a duration of 56 hours. Samples were periodically withdrawn at fixed intervals of 8 hours and subsequently subjected to centrifugation at 9,800g for 12 minutes. The resulting supernatant, devoid of biomass, was analysed for the quantification of total reducing sugars liberated during the enzymatic hydrolysis process.

2.5.2. Enzymatic Hydrolysis of Pretreated SCB Using an Enzyme Cocktail

Partially purified enzyme extracts of *T. harzianum* and *T. viride* were mixed in a 1:1 (v/v) ratio to make an enzyme cocktail for efficient hydrolysis of SCB. The artificial enzymatic cocktail was then utilized for saccharification of pretreated SCB following the methodology described earlier. The saccharification yields of the enzymatic hydrolysis of pretreated SCB were calculated as:

$$\text{Saccharification yield (\%)} = \frac{\text{Reducing sugar obtained} \left[\frac{\text{mg}}{\text{g}} \right]}{\text{Carbohydrate content of the substrate} \left[\frac{\text{mg}}{\text{g}} \right]} \times 100$$

2.6. Fermentation of Sugar Hydrolysates

Sugar hydrolysates with the highest reducing sugar concentrations derived from saccharification with *T. viride* (14.81 g/L), *T. harzianum* (13.69 g/L), and the enzyme cocktail (11.23 g/L) at 15 FPU/g were selected for ethanol fermentation. The fermentation investigations of these sugar hydrolysates were

carried out in screw-capped 250 ml Erlenmeyer flasks, meticulously maintaining semi-anaerobic conditions. To augment the enzymatic hydrolysates, a nutrient mixture comprising malt extract (3.0 g/L), peptone (5.0 g/L), and yeast extract (3.0 g/L) was supplemented, establishing a pH of 6.6 ± 0.2 . Subsequently, a 10.0% (v/v) inoculum of actively growing *Saccharomyces* sp. (optical density OD_{600} 0.6, equivalent to approximately 1×10^7 cells/mL) was introduced under aseptic conditions. The fermentation process was meticulously conducted at $28 \pm 0.5^\circ\text{C}$ over a duration of 30 h. Following this, samples were harvested at fixed intervals of 6 h and subsequently subjected to centrifugation in a cooling centrifuge at a force of 9,800g for 15 min at 4°C . The resulting clarified cell-free supernatants were carefully analyzed to determine the content of residual total reducing sugars and the amount of bioethanol produced (Saini et al., 2020).

Ethanol yield (g/g) = Ethanol produced (g/L) / Sugar consumed (g/L)

2.7. Analytical Procedure

Total reducing sugars were quantified by DNSA colorimetric method using glucose as the calibration standard (Miller, 1959), measured at 540 nm using UV-Visual double-beam spectrophotometer. The quantification of bioethanol was conducted utilizing a spectrophotometric method using the chromic acid oxidation method as described by Caputi and co-workers at 600 nm, with aqueous ethanol as standard (Caputi et al., 1968).

HPLC analysis of sugars and bioethanol were analyzed by using a high-pressure liquid chromatography (HPLC) apparatus (Agilent 1260 infinity, USA) armed with a refractive index detector (RID) and a Zorbax carbohydrate chromatography column (4.6×250 mm, 5 μm particle size). 50 μL of the samples were loaded in a 500 μL sample loop. Samples were run with mobile phase acetone and pure water in a ratio (80:20) at a constant flow rate of 1.0 ml/min and the column oven and detector temperature were kept at 40°C and 35°C , respectively. The retention time (RT) of the samples was compared with the sugar standards (glucose, xylose, cellobiose, mannose, arabinose) at known concentrations for peak identification (Saini et al., 2023).

Bioethanol in fermented broths was confirmed and quantified by using gas chromatography (GC) (Agilent 8860GC) system equipped with a DB-624 capillary column (30 m $\times$ 0.530 mm $\times$ 3.0 μm film thickness) specifically designed for alcohol analysis. The GC was operated with an initial temperature of 200°C , an oven temperature of 70°C (isothermal) and a detector temperature of 300°C . The airflow, hydrogen (H_2) flow, and nitrogen (N_2) flow rates were set at 350 mL/min, 40 mL/min, and 30 mL/min, respectively. The sample flow rate was maintained at 1.5 mL/min. The bioethanol was identified based on its unique retention time and quantified by comparing the peak area with a calibration curve generated using standard ethanol solutions of known concentrations (Saini et al., 2020).

2.8 Mass Balance and Bioethanol Yield Projection

A mass balance calculation was performed to estimate the theoretical bioethanol yield from SCB on a per-hectare basis. Input parameters included: sugarcane productivity of 84 t/ha (Indian national

average); bagasse yield of 30% (w/w) of harvested sugarcane mass; experimental values for glucan content of pretreated SCB (63.76%), saccharification yield (68.12%), fermentation ethanol yield (43.17%), and ethanol density (0.789 g/mL). Each calculation step was cross-validated against experimental data obtained in this study and literature values for comparable processes.

3. Result and Discussion

3.1. Composition Analysis of Two-stage Pretreated SCB

The two-stage hydrothermally pretreated SCB used as substrate in the present investigation was comprehensively characterized by Kumar et al. (2024), whose detailed compositional and structural data serve as the primary benchmark for the downstream processing outcomes reported here. Raw SCB exhibited 38.11% glucan, 23.17% xylan, 19.05% acid-insoluble lignin (AIL), 6.13% acid-soluble lignin (ASL), 5.52% extractives, and 7.02% ash on a dry weight basis. Following optimized two-stage pretreatment (hydrothermal: 190°C, 11.5 min; mechanical disc milling: 34.66 g, 18 s, three passes), the pretreated biomass contained 63.76% glucan, 13.47% xylan, 10.01% AIL, 2.12% ASL, 2.04% ash, and 8.6% extractives—representing a 1.71-fold increase in glucan content, 41.9% xylan removal, and 47.4% reduction in total lignin (AIL + ASL) relative to raw SCB. Solid recovery was 57.9%.

This compositional profile specifically the high glucan content (63.76%) and substantially reduced lignin burden is highly favorable for downstream enzymatic saccharification. The progressive removal of lignin during pretreatment is mechanistically critical: lignin not only physically blocks cellulase access to cellulose microfibrils but also non-productively adsorbs cellulase proteins, reducing effective enzyme concentrations in solution (Sunar et al., 2024). The partial but significant hemicellulose removal (from 23.17% to 13.47% xylan) additionally increases the porosity and swelling capacity of the biomass matrix, further promoting enzyme infiltration. The absence of harsh chemical agents in this pretreatment route means the pretreated biomass slurry required no neutralization or detoxification prior to enzymatic hydrolysis a significant process simplification with direct implications for process economics and inhibitor-free fermentation (Juneja et al., 2020; Sjulander, 2024).

For reference, Table 1 compares the composition of two-stage pretreated SCB from the present study with selected chemical pretreatment benchmarks from Kumar et al. (2024) and literature reports. The two-stage pretreatment achieved superior lignin removal and glucan enrichment compared to all standalone chemical methods evaluated (alkali NaOH: 38.28% lignin removal; sodium chlorite: 33.04%; sulfuric acid: 33.12%; sodium carbonate: 35.30%), while avoiding the chemical waste streams and inhibitor generation associated with those approaches (Kaur and Gaurav, 2024). This positions the two-stage hydrothermal-mechanical pretreatment as the preferred upstream processing strategy for the integrated bioethanol production process described here.

Table 1

Comparative Compositional Analysis of Raw SCB, Two-Stage Pretreated SCB (This Study), and Chemically Pretreated SCB (Kumar et al., 2024) on a Dry Weight Basis.

Pretreatment	Glucan (%)	Xylan (%)	AIL (%)	ASL (%)	Lignin Removal (%)	Glucan Fold-Increase	Solid Recovery (%)
Raw SCB (untreated)	38.11	23.17	19.05	6.13	—	1.00	—
Two-stage (hydrothermal + milling) [†]	63.76	13.47	10.01	2.12	53.01	1.71	57.9
NaOH (1.5%, 121°C, 20 min)	57.11	18.32	12.59	5.14	38.28	1.50	52.0
NaClO ₂ (1.5%, 121°C, 20 min)	56.31	19.14	13.76	5.08	33.04	1.48	66.0
H ₂ SO ₄ (1.5%, 121°C, 20 min)	56.88	12.63	13.67	5.08	33.12	1.49	72.0
Na ₂ CO ₃ (1.5%, 121°C, 20 min)	54.87	18.86	13.32	5.31	35.30	1.44	71.0

[†]Two-stage pretreatment: hydrothermal at 190°C, 11.5 min + wet disc milling (34.66 g biomass, 18 s). AIL = Acid-Insoluble Lignin; ASL = Acid-Soluble Lignin. Data for chemical pretreatments from Kumar et al. (2024).

3.2. Enzyme Production and Partially Purification

The production of cellulases and xylanases was carried out from *T. harzianum* and *T. viride* using wheat bran as a sole carbon source at 75% moisture content under SSF cultures condition. The maximum activities of CMCase (6.33 IU/g) and FPase (8.09 FPU/g) were observed after the 6th day of incubation from *T. viride* under SSF culture condition. Whereas maximum xylanase activity i.e., 9104 IU/g was observed from *T. harzianum* after the 7th day of incubation under SSF culture condition at 30°C. The time-course profiles reflected typical biphasic SSF kinetics an initial growth-associated enzyme induction phase followed by a stationary-phase peak, after which activities declined due to substrate depletion, end-product inhibition, and proteolytic enzyme degradation (Nanjundaswamy and Okeke, 2020; Robak and Balcerek, 2018).

Ammonium sulfate precipitation at 60% saturation substantially concentrated and enriched the enzymatic activities relative to crude extracts. For *T. viride*, specific activities increased to 3.62 IU/mg (FPase), 2.82 IU/mg (CMCase), and 345.07 IU/mg (xylanase), corresponding to fold-increases of 8.63, 9.11, and 6.45, respectively. For *T. harzianum*, fold-increases were 10.87 (FPase), 8.59 (CMCase), and 7.23 (xylanase). These purification factors compare favorably with published reports on *Trichoderma* enzyme systems using wheat bran as substrate (Chutani and Sharma, 2016; Saini et al., 2022) and are consistent with the expected removal of low-molecular-weight non-enzymatic proteins, pigments, and small-molecule contaminants at this purification stage.

The notably higher xylanase activity of *T. harzianum* relative to *T. viride* (9,104 IU/g vs. 9,104 IU/g peak crude; 13,776 vs. 2,437 IU/mL purified) likely reflects strain-specific differences in hemicellulase gene expression profiles and secretome composition, possibly linked to the differential regulation of xylanase-encoding gene families (e.g., GH10, GH11) in these two species (Houfani et al., 2022). Conversely, *T. viride* demonstrated superior overall cellulase activity, suggesting a completer and more active cellobiohydrolase and endoglucanase complement (Kaugarenia et al., 2026). These complementary activity profiles provided the mechanistic rationale for evaluating a combined enzyme cocktail in the saccharification experiments (Table 2).

Table 2
Partial Purification of Cellulases (Cmcase, Fpase) and Xylanase from *T. viride* and *T. harzianum* produced by Solid-State Fermentation on Wheat Bran.

Fungal Culture	Enzyme	Crude Activity (IU/mL)	Crude Sp. Activity (IU/mg)	Purified Activity (IU/mL)	Purified Sp. Activity (IU/mg)	Fold Increase
<i>T. viride</i>	FPase	1.61 ± 0.04	0.42 ± 0.01	17.02 ± 0.35	3.62 ± 0.08	8.63
	CMCase	1.26 ± 0.03	0.31 ± 0.01	12.33 ± 0.28	2.82 ± 0.06	9.11
	Xylanase	220.97 ± 5.2	53.50 ± 1.3	2437.29 ± 48.6	345.07 ± 7.1	6.45
<i>T. harzianum</i>	FPase	1.54 ± 0.04	0.39 ± 0.01	14.07 ± 0.32	4.23 ± 0.09	10.87
	CMCase	0.64 ± 0.02	0.22 ± 0.01	6.96 ± 0.18	1.88 ± 0.05	8.59
	Xylanase	1820.97 ± 41.3	309.16 ± 7.2	13776.35 ± 295.4	2235.22 ± 48.1	7.23

Values are mean ± standard deviation (n = 3). IU = International Units; Sp. Activity = Specific Activity; FPase = Filter Paper cellulase; CMCase = Carboxymethyl cellulase activity.

3.3. Enzymatic Saccharification of Pretreated SCB

The saccharification of two-stage pretreated SCB was conducted using crude enzymes from *T. harzianum* and *T. viride* individually and in combination, across enzyme loadings of 5–20 FPU/g and a hydrolysis duration of up to 56 h. The temporal evolution of total reducing sugar (TRS) release as a function of enzyme loading and fungal source is summarised in Fig. 1.

Saccharification with *T. harzianum* cellulase has shown a clear positive correlation between enzyme loading and TRS yield up to 40 h of incubation (Fig. 1a). At the optimal loading of 15 FPU/g, TRS release reached 387.32 mg/g after 40 h, with the yield plateau thereafter attributable to end-product inhibition by accumulated glucose and cellobiose on β-glucosidase and exoglucanase activities. (Kumar et al., 2023; Liu et al., 2025a). Increasing the enzyme dosage beyond 15 FPU/g to 20 FPU/g produced only marginal improvement, indicating that substrate availability and not enzyme quantity became the rate-limiting factor at the higher loading, consistent with a substrate saturation effect in heterogeneous enzymatic

systems (Ike et al., 2025a). Saccharification with 15 FPU/g of substrate was selected as an optimum condition for enzymatic solubilization.

T. viride cellulases demonstrated superior saccharification performance across all enzyme loadings tested (Fig. 1b) (Kumar et al., 2024b). At the optimal 15 FPU/g, TRS release reached 417.40 mg/g after 40 h—7.77% higher than *T. harzianum* at the same loading reflecting the stronger cellulolytic cocktail activity (higher FPase and CMCase) of this strain (Ali et al., 2026). The saccharification yield at optimum conditions was 68.12%, calculated on the basis of the glucan and xylan content of the pretreated substrate. The enzyme cocktail prepared from equal volumes of *T. viride* and *T. harzianum* extracts yielded the highest absolute TRS release at the maximum loading: 423.88 mg/g at 20 FPU/g (Fig. 1c) (Sharma et al., 2025), representing a 1.5% and 9.5% improvement over *T. viride* and *T. harzianum* individually, respectively. The synergistic action of the cocktail likely arose from the complementary cellulase:xylanase activity ratio *T. harzianum* contributing disproportionately higher xylanase activity that facilitated hemicellulose hydrolysis and thereby further increased cellulose accessibility, consistent with the known synergism between xylanases and cellulases in the deconstruction of hemicellulose-cellulose composites (Kaugarenia et al., 2026; Saini et al., 2020).

Table 3 summarizes saccharification outcomes across all enzyme systems and compares them with selected literature benchmarks for SCB enzymatic hydrolysis. The TRS yields obtained in the present study (387.32–423.88 mg/g) are competitive with and, in several cases, exceed those reported for commercially saccharified SCB using conventional chemical pretreatment (180–380 mg/g) (Kaur and Gaurav, 2024), while benefiting from a chemical-free pretreatment upstream that avoids fermentation inhibitor generation.

Table 3

Summary of Enzymatic Saccharification Performance of Two-Stage Pretreated SCB Under Optimized Conditions (15 FPU/g, 40 h, 50°C, pH 5.0, 5% w/v substrate consistency).

Enzyme System	Optimal Loading (FPU/g)	TRS Released (mg/g)	Saccharification Yield (%)†	Comparison vs. Control (fold)
<i>T. viride</i> cellulases	15	417.40 ± 8.3	68.12	13.8
<i>T. harzianum</i> cellulases	15	387.32 ± 7.6	63.16	12.8
Enzyme cocktail (1:1 v/v)	20	423.88 ± 9.1	69.14	14.0
Unaccharified control	—	30.25 ± 1.2	—	1.0

†Saccharification yield = (TRS released / total carbohydrate content of pretreated SCB) × 100. Control = pretreated SCB without enzyme addition. Values are mean ± SD (n = 3).

3.4. HPLC Analysis of Sugar Hydrolysates

HPLC chromatographic profiling of enzymatic hydrolysates was performed to qualitatively and quantitatively characterize the sugar composition released during saccharification of pretreated SCB. The chromatograms of enzymatic hydrolysates (*T. harzianum*, *T. viride*, and enzyme cocktail) saccharified samples all exhibited a prominent glucose peak at approximately 7.02 minutes, confirming successful cellulose hydrolysis post-enzymatic treatment (Fig. 2) (Ruan et al., 2024). Conversely, the chromatogram of untreated and unsaccharified biomass (control) displayed an absence of the glucose peak, confirming the absence of substrates for enzymatic hydrolysis. Notably, intermediates of cellulose hydrolysis, including cellobiose (RT $\approx$ 8.0 min), indicate incomplete β -glucosidase processing of cellobiose to glucose, consistent with the known rate-limiting nature of β -glucosidase in *Trichoderma* cellulase systems and explaining the slight yield improvement seen with the enzyme cocktail, which may have better balanced exo- and endo-glucanase activities (Liu et al., 2025b). Furthermore, the enzymatic hydrolysis of SCB revealed the appearance of hemicellulose hydrolysis products such as xylose (RT $\approx$ 5.6 min), confirming the activity of the xylanase component of both enzyme preparations (Chen et al., 2024). These peaks were conspicuously absent in the chromatogram of the unsaccharified samples, confirming baseline specificity and the absence of contaminating monosaccharides in the pretreated substrate (Saini et al., 2022).

The simultaneous release of glucose, cellobiose, and xylose substantiates the synergistic deconstruction of both the cellulosic and hemicellulosic fractions of the two-stage pretreated SCB by the fungal enzyme systems (Ike et al., 2025b). The detection of xylose is particularly noteworthy from a biorefinery perspective: if co-fermented alongside glucose by xylose-fermenting organisms (e.g., *Scheffersomyces stipitis* or engineered *Saccharomyces cerevisiae* strains), this pentose fraction could increase total ethanol yields by an estimated 20–30%, representing a significant untapped opportunity in the current process (Hretskyi et al., 2025a, 2025b; Singh et al., 2024; Zhang et al., 2025).

3.5. Bioethanol Production from SCB Hydrolysates

Ethanol production from SCB sugar hydrolysates employing diverse catalysts, including *T. harzianum*, *T. viride* and enzyme cocktail, shedding light on versatile bioconversion strategies. Ethanol production from sugar hydrolysates of SCB saccharified, employing cellulases from *T. harzianum* was evaluated at distinct time intervals (0, 6, 12, 18, 24, and 30 h). Commencing with an initial sugar concentration of 13.69 g/L, an initial ethanol yield of 0.07 g/g and 7.07% was observed (Fig. 3a) (Ali et al., 2026). Subsequently, a noteworthy progression revealed a remarkable decrease in sugar concentration (0.95 g/L) by 24 h, accompanied by a substantial ethanol production of 5.80 g/L. This outcome corresponded to an enhanced ethanol yield of 0.42 g/g and 42.42% (Table 4).

Similarly, ethanol production from sugar hydrolysates of SCB saccharified employing cellulases from *T. viride* was studied over different time intervals (0, 6, 12, 18, 24, and 30 h). At the onset, with an initial sugar concentration of 14.81 g/L, an initial ethanol yield of 0.09 g/g and 9.50% was observed (Fig. 3b). As time advanced, the fermentation process exhibited notable enhancement. The ensuing time intervals revealed a continuous reduction in sugar concentration, accompanied by a remarkable increase in

ethanol production and yield. At the 24 h, with a sugar concentration of 1.03 g/L, a remarkable ethanol production of 6.39 g/L was achieved (Fig. 3b), yielding 0.43 g/g and 43.17% ethanol yield (Table 4) (Ali et al., 2026; Kaur and Gaurav, 2024) .

Further, the ethanol production from sugar hydrolysates of SCB saccharified, employing cellulases from *T. viride* and *T. harzianum* revealed a consistent trend of increased ethanol production and yield (Fig. 3c). At the initial stage, with an initial sugar concentration of 11.23 g/L, an ethanol yield of 0.11 g/g (11.23%), progressing to an impressive ethanol yield of 0.38 g/g (38.65%) at 24 h, corresponding to a sugar concentration of 0.96 g/L (Fig. 3c). This dynamic transformation emphasized the efficacy of the cocktail in enhancing sugar concentration for ethanol production. The fluctuating ethanol production rate showcased the temporal interplay of substrate conversion and product formation. These findings collectively accentuate the potential of (Kumar et al., 2024b), presenting a promising strategy for enzymatic hydrolysis and efficient ethanol production from LCB.

Table 4

Summary of Bioethanol Production from Enzymatic Hydrolysates of Two-Stage Pretreated SCB by *Saccharomyces* Sp. Under Optimized Fermentation Conditions (28°C, pH 6.6, 30 h, 10% v/v inoculum).

Enzyme System	Initial Sugar (g/L)	Residual Sugar at 24 h (g/L)	Ethanol at 24 h (g/L)	Ethanol Yield (g/g)	Fermentation Efficiency (%)	% Theoretical Yield
<i>T. viride</i> cellulases	14.81	1.03	6.39 ± 0.12	0.43	43.17	86.34
<i>T. harzianum</i> cellulases	13.69	0.95	5.80 ± 0.11	0.42	42.42	82.96
Enzyme cocktail (1:1 v/v)	11.23	0.96	4.34 ± 0.09	0.38	38.65	75.64

Theoretical maximum yield = 0.511 g ethanol/g glucose (Gay-Lussac stoichiometry). % Theoretical yield = (Observed ethanol yield / 0.511) × 100. Values are mean ± SD (n = 3).

3.6. GC Analysis of Bioethanol from Fermented Broth

Gas chromatographic analysis of fermented broths confirmed the presence of bioethanol in all three hydrolysate fermentation systems (Fig. 4).

The chromatograph of fermented broth exhibited a characteristic ethanol peak for hydrolysate of *T. harzianum* (RT 3.268 min) (Fig. 4a), *T. viride* (RT 3.298 min) (Fig. 4b), and (c) an enzymatic cocktail (RT 3.252 min) (Fig. 4c). The minor variations in retention time are consistent with normal chromatographic drift within the ± 0.05 min tolerance window and do not indicate a different chemical identity. Ethanol identity was confirmed by comparison with the calibration standard retention time and by the absence of the ethanol peak in unfermented (enzyme-free or *Saccharomyces*-free) control chromatograms. Peak area integration across the five-point calibration curve corroborated the spectrophotometric ethanol

quantification values, with deviations of less than 5% between methods, confirming analytical method consistency.

The results indicated that the *T. viride* produced the highest bioethanol yield, followed by *T. harzianum*, and enzymatic cocktail, in decreasing order of efficiency. (Kumar et al., 2024b; Raheja et al., 2025; Sharma et al., 2025). These results were confirmed by GC quantification and are consistent with the saccharification yield data, reinforcing the conclusion that upstream enzymatic saccharification efficiency is the primary driver of downstream fermentation output in this integrated process. These findings collectively demonstrate the suitability of the GC method (DB-624 column, FID detector) as a reliable, independent validation tool for bioethanol produced from lignocellulosic hydrolysates (Syazwanee, 2021). This research provides valuable insights into the potential of SCB as a feedstock for bioethanol production and emphasizes the critical importance of selecting the most appropriate cellulase for the saccharification process. This information is pivotal for optimizing bioethanol production from SCB and advancing the sustainability of biofuel production.

3.7 Mass Balance and Industrial Bioethanol Yield Projection

A mass balance analysis was performed to translate laboratory-scale process efficiencies into projected industrial-scale bioethanol yields and to contextualize the results within the agro-economic framework of Indian sugarcane production (Table 5). The calculation proceeded as follows: India's national average sugarcane productivity is approximately 84 metric tonnes (t) per hectare per growing season, and SCB constitutes approximately 30% (w/w) of harvested cane mass following juice extraction (Kar et al., 2023; Misra et al., 2026). Each hectare therefore yields $84 \times 0.30 = 25.2$ t SCB. Of the dry mass of pretreated SCB, 63.76% is glucan (carbohydrate available for saccharification). At a saccharification yield of 68.12% (corresponding to 417.40 mg TRS per g substrate), 25.2 t SCB yields approximately 10.94 t fermentable sugars. At the observed fermentation efficiency of 43.17% (ethanol yield 0.43 g/g), fermentation of 10.94 t sugars produces approximately 4.71 t ethanol. Given the density of ethanol at 20°C of 0.789 kg/L (Rebolledo-Leiva et al., 2026), this is equivalent to approximately 5,968 L of ethanol/hectare from raw biomass processing.

However, accounting for the solid recovery of 57.9% through the two-stage pretreatment (i.e., 25.2 t SCB input $\rightarrow$ 14.6 t pretreated biomass), and applying saccharification and fermentation efficiencies to the pretreated biomass mass, the projected ethanol yield is 3,263 L/ha—equivalent to approximately 25.74 MJ/ha of energy delivered as liquid fuel. This projection is competitive with reported bioethanol yields from conventionally pretreated SCB (1,800–4,500 L/ha in the literature, depending on pretreatment and fermentation efficiency) and substantially superior to thermal energy recovery from direct SCB combustion ($\sim 8,000$ MJ/ha at 19.6 MJ/kg), particularly when the higher economic value of liquid bioethanol and avoided fossil fuel displacement are considered (Bakali and Wycliffe, n.d.; Garriga et al., 2024; Xian et al., 2024).

Table 5
Mass Balance and Projected Bioethanol Yield Per Hectare of Sugarcane Cultivation.

Parameter	Value	Basis / Unit
Sugarcane productivity	84.0	t/ha (Indian national average)
SCB yield from sugarcane	25.2	t/ha (30% w/w of cane mass)
Solid recovery after two-stage pretreatment	57.9%	Experimental; Kumar et al., 2024
Pretreated SCB available for hydrolysis	14.60	t/ha
Glucan content of pretreated SCB	63.76%	Experimental (dry weight basis)
Saccharification yield at 15 FPU/g, 40 h	68.12%	This study (<i>T. viride</i> cellulases)
Fermentable sugars released	6.34	t/ha
Fermentation ethanol yield	0.43 g/g	This study (<i>Saccharomyces</i> sp., 24 h)
Bioethanol produced (mass)	2.73	t/ha
Bioethanol produced (volume)	~ 3,263	L/ha (density 0.789 kg/L)
Energy equivalent	~ 25.74	GJ/ha

4. Conclusion

The present investigation establishes a fully integrated, chemical-free bioethanol production pathway from sugarcane bagasse that spans two-stage hydrothermal-mechanical pretreatment, in-house fungal enzyme production, enzymatic saccharification, and yeast fermentation within a coherent experimental framework. The two-stage pretreatment of SCB optimized by Kumar et al. (2024) and employed here as the upstream process achieved 53.01% lignin removal with 1.71-fold glucan enrichment, surpassing all conventional chemical methods evaluated, without generating fermentation inhibitors or chemical waste streams. In-house production of cellulolytic and xylanolytic enzymes from *Trichoderma viride* and *Trichoderma harzianum* under wheat bran-based solid-state fermentation achieved economically relevant enzyme titers, with partial ammonium sulfate purification yielding fold-increases of 8.63–10.87 for FPase across both strains.

Enzymatic saccharification of pretreated SCB at 15 FPU/g for 40 h released 417.40 mg/g total reducing sugars with *T. viride* cellulases (saccharification yield: 68.12%), while an artificial enzyme cocktail at 20 FPU/g achieved 423.88 mg/g through synergistic cellulose-hemicellulose deconstruction. HPLC profiling confirmed the simultaneous release of glucose, cellobiose, and xylose from the pretreated substrate, demonstrating the versatility of the enzymatic system in mobilizing both hexose and pentose fractions. Fermentation of *T. viride* hydrolysates by *Saccharomyces* sp. produced 6.39 g/L bioethanol at 86.34% of the theoretical maximum confirming that the chemical-free pretreatment strategy yields hydrolysates free of significant fermentation inhibitors. Mass balance analysis projected a bioethanol yield of

approximately 3,263 L per hectare of sugarcane cultivation under Indian agricultural productivity conditions.

These findings collectively support the environmental and economic viability of the two-stage hydrothermal-mechanical pretreatment platform as a foundation for lignocellulosic biorefinery operations in sugarcane-producing regions. Future work should address: (i) co-fermentation strategies for xylose utilization to maximize pentose-derived ethanol yields; (ii) simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) approaches to reduce process steps and capital costs; (iii) scale-up of enzyme production and saccharification to pilot scale; (iv) comprehensive techno-economic analysis (TEA) and life cycle assessment (LCA) of the integrated process; and (v) valorization of the lignin-enriched pretreatment liquor as a co-product for phenolic chemicals or bioenergy to complete the circular bioeconomy vision.

Novelty Statement

This study presents the first fully integrated process account from chemical-free two-stage hydrothermal-mechanical pretreatment through in-house fungal enzyme production, optimized enzymatic saccharification, and fermentation applied to sugarcane bagasse, anchored to a rigorously validated mass balance framework. The combined pretreatment strategy achieves 53.01% lignin removal without chemical inputs or inhibitor generation, directly enabling an 86.34% fermentation efficiency relative to the theoretical maximum. The comparative multi-enzyme system evaluation (*T. viride*, *T. harzianum*, and their cocktail) provides a mechanistic understanding of the enzyme synergy that drives saccharification efficiency gains. The projected 3,263 L bioethanol/ha provides a quantitative benchmark for the techno-economic assessment of this eco-friendly biorefinery pathway in the Indian agricultural context.

Declarations

The authors declare no conflicts of interest relevant to this work. No financial, personal, or professional relationships have influenced the preparation or submission of this manuscript.

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Author Contribution

Ravi Kumar: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Writing - original draft. Nitin Kumar: Conceptualization; Formal analysis; Investigation; Project administration; Resources; Validation; Visualization; Writing - review & editing. Gaurav Chaudhary: Supervision; Data

curation; Formal analysis; Investigation; Methodology; Validation. Sonu Saini: Data curation; Formal analysis; Investigation; Methodology; Software; Writing - review & editing. Charan Singh: Methodology; Software.

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Any additional data will be made available on reasonable request.

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Figures

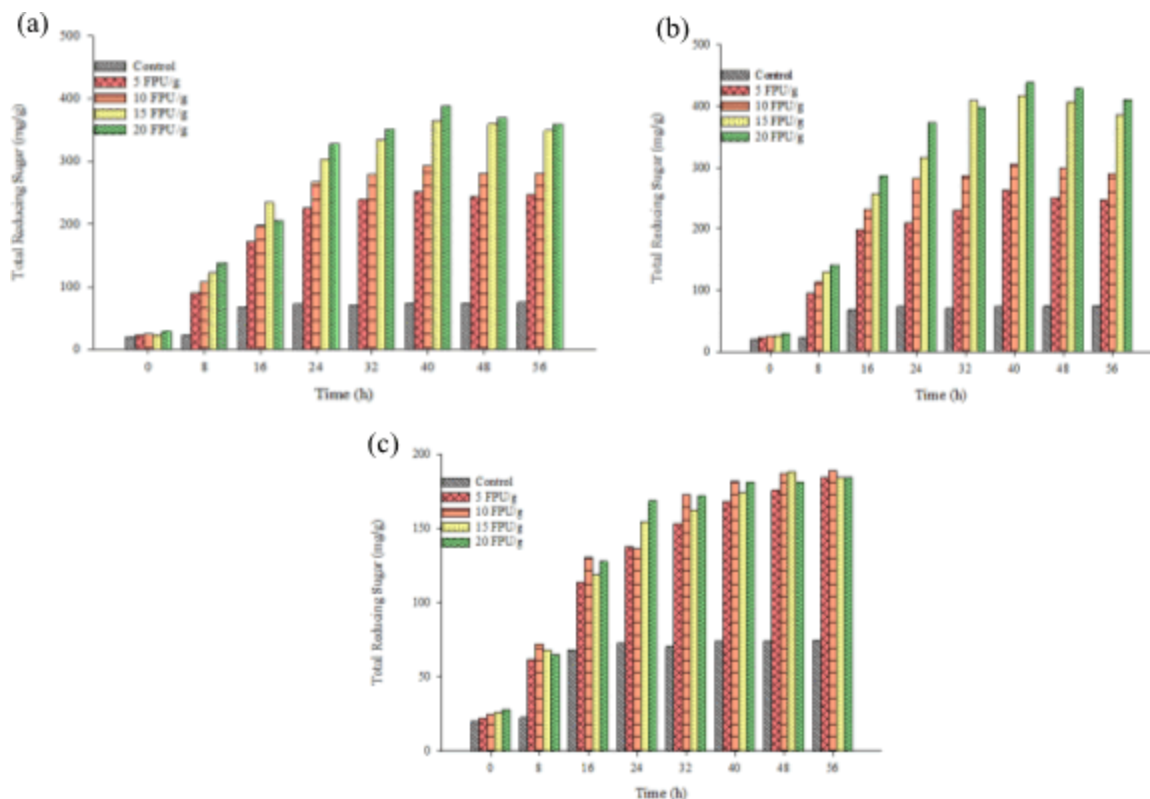


Figure 1

Enzymatic Hydrolysis of Pretreated SCB Crude Cellulases Extracted from (a) *T. harzianum*, (b) *T. viride*, and (c) enzyme cocktail of *T. viride* and *T. harzianum*.

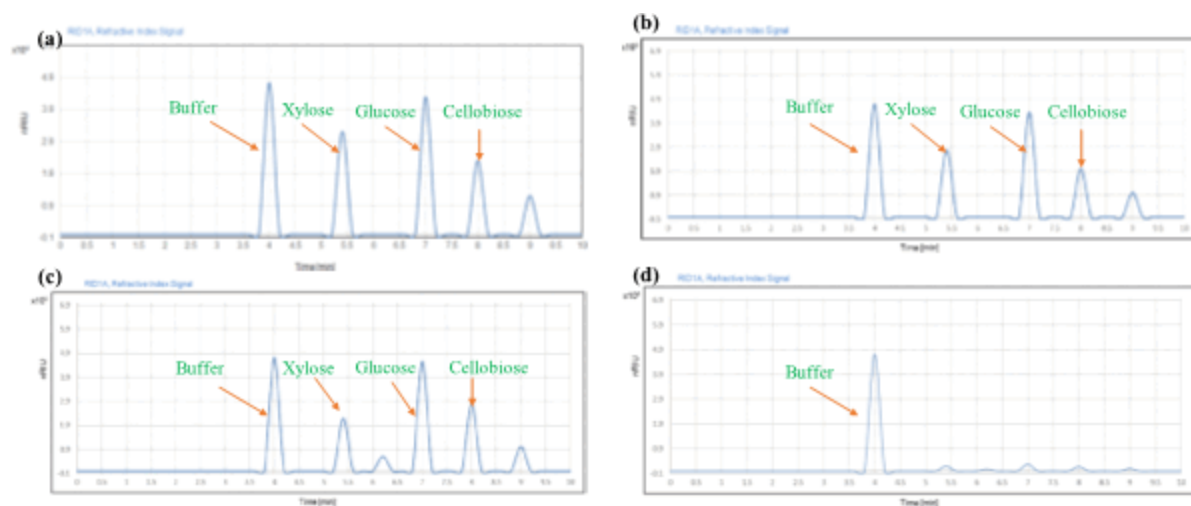


Figure 2

HPLC Analysis of Sugar Hydrolysates of Combinedly Pretreated SCB. Biomass Saccharified Using Cellulases from (a) *T. harzianum*, (b) *T. viride*, (c) Enzymatic cocktail, (d) Unsaccharified biomass

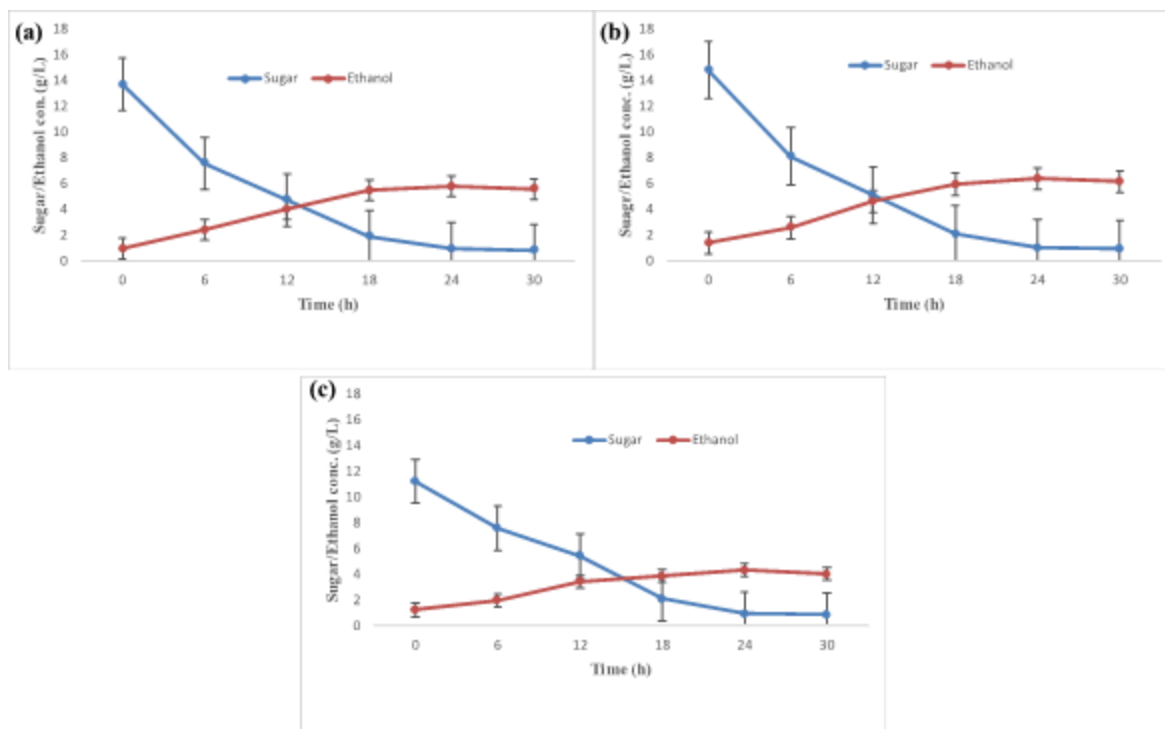


Figure 3

Bioethanol Production from Sugar Hydrolysates of SCB. Saccharified Using Cellulases from (a) *T. harzianum*, (b) *T. viride*, and (c) an enzymatic cocktail.

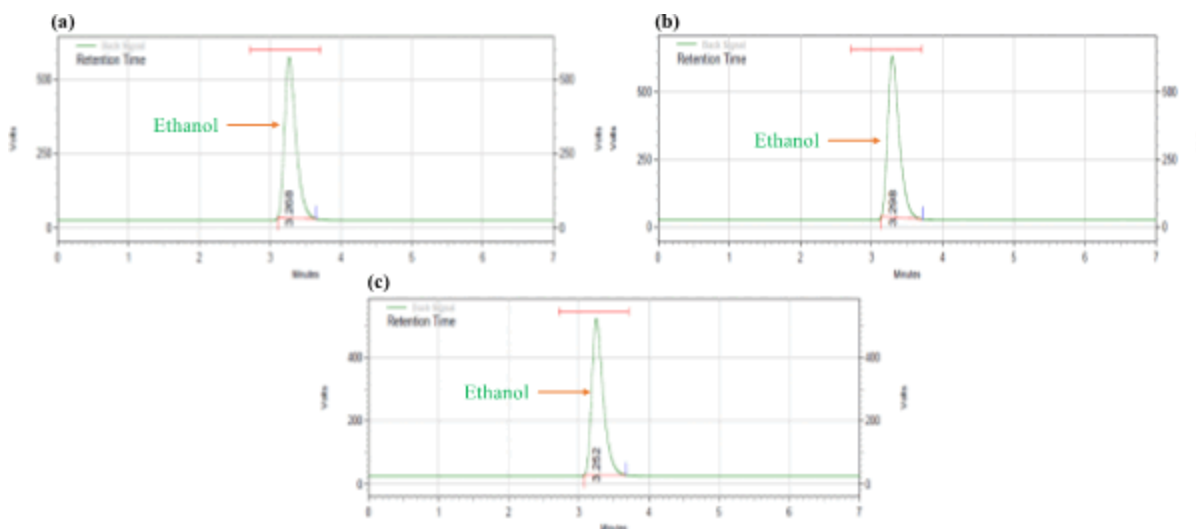


Figure 4

Chromatographic Analysis of Bioethanol from Sugar Hydrolysates of SCB. Saccharified Product Using Cellulases from (a) *T. harzianum*, (b) *T. viride*, and (c) enzymatic cocktail

Supplementary Files

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