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Enhanced Saccharification Yield of Alkali Pretreated Sugarcane Bagasse Utilizing Customized Cellulase Cocktail from *Trichoderma harzianum* and *Trichoderma viride*

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Abstract

The experiment was conducted during 2023 at Bioenergy Laboratory, Department of RBEE, College of Agricultural Engineering and Technology, CCSHAU, Hisar, Haryana, India. Compositional changes in sugarcane bagasse subjected to varying concentrations of sodium hydroxide (0.3% to 1.2%), revealing significant increases in glucan content (from 37.13% to 53.81%) alongside decreases in xylan, acid-insoluble lignin, acid-soluble lignin, ash, and other extractives. These changes were validated using microscopic technique SEM, confirming the efficacy of the pretreatment process. The utilization of a customized cellulase cocktail derived from *Trichoderma harzianum* and *Trichoderma viride* holds significant promise in enhancing the saccharification from alkali-pretreated sugarcane bagasse. This study investigates the synergistic effects of cellulase enzymes produced by these fungi on the hydrolysis of lignocellulosic biomass. The enzymatic hydrolysis process is optimized by varying enzyme dosages, reaction conditions, and incubation times to maximize the release of fermentable sugars. Results indicate a substantial improvement in saccharification efficiency with the customized cellulase cocktail, highlighting its potential for sustainable biofuel production. The pretreated sugarcane bagasse, when saccharified with *Trichoderma harzianum* and *Trichoderma viride* individually, released 254.43 mg g^{-1} and 325.53 mg g^{-1} of reducing sugars, respectively, after 40 h of incubation. In contrast, the combined enzymatic cocktail achieved a substantial increase in glucose yields (345.12 mg g^{-1}) at 40 h, showcasing the synergistic effect of the combined enzymatic activity. This research contributes to advancing bioconversion technologies for utilizing lignocellulosic biomass resources efficiently and economically, thus addressing key challenges in sustainable energy production.

Keywords: Cellulase, enzyme, reducing sugar, sugarcane bagasse, saccharification

1. Introduction

The global energy landscape is undergoing a profound transformation, driven by the urgent need to mitigate climate change, reduce reliance on finite fossil fuel reserves, and foster sustainable development (Ajala et al., 2021; Banyal et al., 2022). In this context, the quest for renewable and environmentally friendly energy sources has become increasingly imperative. Among the array of renewable energy options, biofuels hold significant promise due to their potential to replace conventional fossil fuels while mitigating greenhouse gas emissions and promoting rural development (Baksi et al., 2023; Saini et al., 2023).

Lignocellulosic biomass, comprising cellulose, hemicellulose, and lignin, stands out as a prominent feedstock for biofuel production owing to its abundance, wide distribution, and renewable nature (Chen et al., 2022). Among the diverse sources of lignocellulosic biomass, sugarcane bagasse emerges as a particularly attractive substrate due to its high cellulose content and widespread availability as a byproduct of the sugar industry (Kumar et al., 2024). Each year, millions of tons of sugarcane bagasse are generated globally, presenting a vast resource for bioenergy production (Rana et al., 2023; Halysh et al., 2020).

However, the efficient conversion of lignocellulosic biomass into fermentable sugars, a crucial step in biofuel production, presents formidable challenges (Saini et al., 2023). The recalcitrant nature of lignocellulose, characterized by its complex and rigid structure, poses a significant barrier to enzymatic hydrolysis, the process by which polysaccharides are broken down into simple sugars (Baksi et al., 2023). To overcome this challenge, various pretreatment strategies have been developed to disrupt the lignocellulosic matrix, thereby enhancing the accessibility of cellulose to hydrolytic enzymes (Brienzo et al., 2016; Guragain et al., 2016). By disrupting the

intermolecular bonds within lignocellulose and increasing the porosity of the biomass substrate, alkali pretreatment facilitates the enzymatic hydrolysis of cellulose, leading to higher sugar yields (Kundu et al., 2023). However, despite the significant advancements in pretreatment technologies, achieving high saccharification yields from lignocellulosic biomass remains a formidable challenge that hampers the commercial viability of biofuel production (Kaur and Kuhad, 2019; Kumar and Vatsa, 2024).

Microbial enzymes, particularly those derived from filamentous fungi, have attracted considerable attention as potent biocatalysts for lignocellulose saccharification. Filamentous fungi possess a diverse array of cellulolytic, and hemicellulolytic, which act synergistically to depolymerize the complex structure of lignocellulose into fermentable sugars (Chakraborty et al., 2016). Previously, *Trichoderma* species especially *Trichoderma* species, *Trichoderma harzianum* and *Trichoderma viride* have emerged as key candidates for bioconversion processes due to their robust enzyme secretion machinery and broad substrate specificity (Nanjundaswamy and Okeke, 2020; Pathak et al., 2014).

While individual strains of *Trichoderma* have shown promising saccharification capabilities, the potential synergy arising from the combination of different species remains relatively unexplored (De Oliveira Rodrigues et al., 2022). Synergistic interactions between microbial consortia have been documented in various ecological niches, leading to enhanced metabolic activities and ecological fitness. Leveraging such synergies in lignocellulose degradation could offer a novel approach to improve saccharification efficiency and reduce the costs associated with biofuel production (Chakraborty et al., 2016).

In this study, we aimed to investigate the cooperative interactions between cellulases from *Trichoderma harzianum* and *Trichoderma viride* in the saccharification of alkali-pretreated sugarcane bagasse. We hypothesized that the combined action of these two fungal species would result in a synergistic enhancement of enzymatic hydrolysis efficiency, thereby improving the overall yield of fermentable sugars. By elucidating the mechanisms underlying this synergistic effect, we aim to contribute to the development of more efficient and sustainable strategies for lignocellulosic biofuel production.

2. Materials and Methods

The current research work was carried out in 2022–23, department of processing and food engineering, CCSHAU Hisar, Haryana, India. Hisar, a city in Haryana, is situated at 29.14°N, 75.72°E, at an elevation of 224 metres above sea level.

2.1. Chemicals and reagents

All the reagents and chemicals utilized in the current study were of analytical grade available commercially. Specific chemicals like 3, 5-dinitro salicylic acid (DNSA) were procured from Sigma-Aldrich (US). Sodium hydroxide, sodium sulfite, phenol, glucose, xylose and sodium potassium tartrate were obtained from Hi-Media (India).

2.2. Lignocellulosic biomass material

The alkali (NaOH) pretreated sugarcane bagasse biomass was obtained from department of processing and food engineering, CCSHAU Hisar, Haryana, India. The pretreated biomass was stored in air tight bags to protect the biomass from moisture, insect and pests (Kumar et al., 2024).

2.3. Compositional analysis of lignocellulosic biomass

The compositional analysis was conducted to examine the different structural constituents of untreated and pretreated sugarcane bagasse. This analysis involved assessing the biomass components, including cellulose (glucan), hemicellulose (xylan), lignin (acid-insoluble lignin (AIL) and acid soluble lignin (ASL)), and ash content, using a method based on the National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008), with slight modification. The determination of lignin content followed the procedures outlined in the laboratory analytical procedures (LAPs) provided by NREL.

2.4. Alkali pretreatment of sugarcane bagasse

Sugarcane bagasse underwent pretreatment with sodium hydroxide, maintaining a 10% (w/v) consistency. The biomass was treated with varying alkali concentrations, ranging from 0.3% to 1.2% (w/v), then subjected to autoclave at 121°C for 20 minutes. Following the pretreatment, the alkali-pretreated sugarcane biomass was separated by filtration using an eight-layered muslin cloth and washed with running tap water until it achieved a neutral pH. The filtrate was subsequently separated via centrifugation at 10,000g for 12 minutes. The resulting filtrate, devoid of biomass, underwent testing to determine the levels of total reducing sugars and released phenols (Saini et al., 2023). The solid residues that remained underwent was comprehensively analysed for its composition, following the modified NREL method (Sluiter et al., 2008).

2.5. Fungal enzymes

Crude fungal cellulases produced from *Trichoderma harzianum* and *Trichoderma viride* species were obtained from the Bioenergy Laboratory, Department of RBEE, College of Agricultural Engineering and Technology, CCSHAU, Hisar-125004, Haryana, India. Enzymes were stored at 4 °C in a refrigerator after centrifugation until further use (Kumar et al., 2024; Saini et al., 2023).

2.6. Enzymatic hydrolysis of pretreated SCB using cellulase

Cellulases derived from the fungi *T. harzianum* and *T. viride* were separately used to break down pretreated sugarcane bagasse (SCB) following alkali pretreatment. They carried out the enzymatic hydrolysis of the delignified biomass at a consistency of 5% (w/v) with 50 mM acetate buffer at pH 5.0. This buffer was enriched with 0.5% Tween-80 and 0.005% sodium azide. In a 250 ml Erlenmeyer flask, 2.5 g of pretreated

SCB was mixed with 50 ml of the buffer solution, along with 250 µl of Tween-80 and 0.0025 g of sodium azide. The biomass was conditioned before enzyme addition by incubating it at 50 ºC and 150 rpm for 2 h in a shaking incubator. Then, the cocktail enzymes were added at doses ranging from 5 to 20 FPU g^{-1} of the substrate, and the reaction was allowed to proceed at 50°C for 56 h. At fixed intervals of 8 h, samples were withdrawn and subjected to centrifugation at 9,800 g for 12 minutes (Saini et al., 2022). The resulting supernatant, devoid of biomass, underwent analysis to quantify the total reducing sugars liberated during the enzymatic hydrolysis process.

2.7. Enzymatic saccharification of pretreated SCB using customized cellulase cocktail

Cellulases from the ascomycetous fungi, *T. harzianum* and *T. viride,* were mixed in a 1:1 (v/v) ratio to produce an enzyme cocktail for enhancing the hydrolysis of SCB pretreated with an alkali pretreatment method. The subsequent procedure closely followed the methodologies outlined in Section 2.4.

The experimental workflow was depicted graphically in a flowchart format (Figure 1), outlining the sequential steps involved in the alkali pretreatment and enzymatic hydrolysis of sugarcane bagasse biomass.

3. Results and Discussion

3.1. Raw sugarcane bagasse characterization

Sugarcane bagasse exhibits a composition of 37.13±0.81% glucan, 21.89±0.24% xylan, 17.17±0.63% acid-insoluble lignin (AIL), 6.59±0.18% acid-soluble lignin (ASL), 9.12±0.11% extractives, and 8.10±0.24% ash on a dry weight basis. Previous investigations have found the native biochemical compositions of sugarcane bagasse as lignin (34±4.3%), hemicellulose (22±2.7%), cellulose (34±2.1%), and other extractives (11±0.9%) on a dry weight basis (Halysh et al., 2020; Kumar et al., 2024). Inconsistencies in sugarcane bagasse composition across studies may arise from environmental factors, geographical variations, differences in dietary habits, and the absence of standardized protocols for assessing its inherent chemical composition.

3.2. Alkali pretreatment sugarcane bagasse

Numerous experiments were conducted to determine the optimal alkali concentration for the breakdown of lignin in sugarcane bagasse material. The impact of various alkali agents on lignin degradation and glucan increment was evaluated (Figure 1). A direct correlation was observed between the concentration of sodium hydroxide and the removal of lignin (Plate 1). As the NaOH concentration increases from 0.3% to 1.2%, there is a notable increase in glucan content from 37.13% to 53.81%, indicating enhanced breakdown of structural components and potential enhancement of cellulose availability. Conversely, xylan content decreases gradually from 21.89% to 16.94% with increasing NaOH concentration, suggesting the partial breakdown of hemicellulose under alkaline conditions.

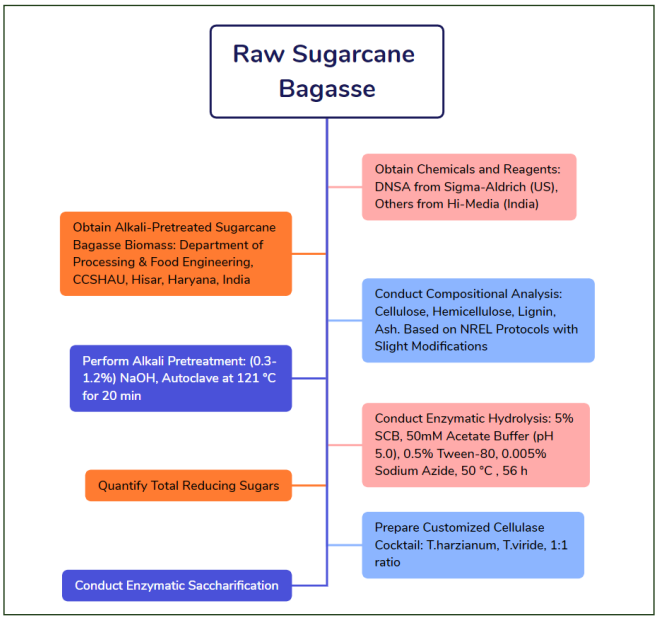

Figure 1: Schematic representation of the experimental workflow for alkali pretreatment and enzymatic hydrolysis of sugarcane bagasse biomass

Plate 1: Pretreatment of sugarcane bagasse with different concentrations

The AIL and ASL contents also decrease with increasing NaOH concentration, indicating effective lignin removal. Specifically, AIL decreases from 17.17% to 13.55%, while ASL decreases from 6.59% to 3.08%. This suggests that higher NaOH concentrations lead to more efficient lignin breakdown. Moreover, the ash content decreases from 8.10% to 4.11% as the NaOH concentration increases, likely due to the removal of mineral impurities during the alkali treatment process. Interestingly, the content of other extractives (OE) fluctuates inconsistently across different NaOH concentrations, indicating potential variations in non-structural components. Overall, the results demonstrate that increasing NaOH concentration leads to significant changes in the composition of sugarcane bagasse, with notable impacts on cellulose, hemicellulose, lignin, and ash contents. The impact of sodium hydroxide pretreatment on sugarcane bagasse surface morphology was observed through SEM microphotographs (Figure 1).

NaOH recognized for its potent alkalinity, initiates the breakdown of lignin through a process termed saponification. This mechanism involves the cleavage of ester and ether bonds within lignin, resulting in the solubilization of lignin and its partial removal from the biomass matrix (Saini et al., 2023).

Consequently, this process yields a lignin-depleted biomass enriched in cellulose (Kumar et al., 2024). Furthermore, in alkaline environments, sodium hydroxide facilitates the hydrolysis of glycosidic bonds within hemicellulose (Guragain et al., 2016), leading to the liberation of monomeric sugars such as xylose, mannose, and arabinose. This hydrolytic action contributes to the alteration of the biomass composition, enabling the release of potentially fermentable sugars for downstream bioprocessing applications.

3.3. Sem analysis of control and pretreated sugarcane bagasse

Microstructural alterations induced by alkali pretreatment under optimized conditions was conducted using FESEM to elucidate the cell wall characteristics of sugarcane bagasse. The surface morphology of untreated biomass exhibited a compact, rigid, and regularly organized structure (Figure 2(a)), attributed to the intact and tightly bound lignin sheath enveloping the carbohydrate matrix of SCB biomass. The application of NaOH facilitated the disintegration of lignin structures, resulting in a smoother surface texture resembling pulp (Figure 2(b)). These pretreatment strategies led to substantial lignin removal, consequently imparting a highly porous architecture to the biomass. This enhanced porosity could potentially facilitate improved accessibility to cellulose and hemicellulose fractions within the biomass matrix, thereby enhancing enzymatic digestibility and subsequent bioconversion processes for sustainable biorefinery applications (Saini et al., 2023) (Table 1).

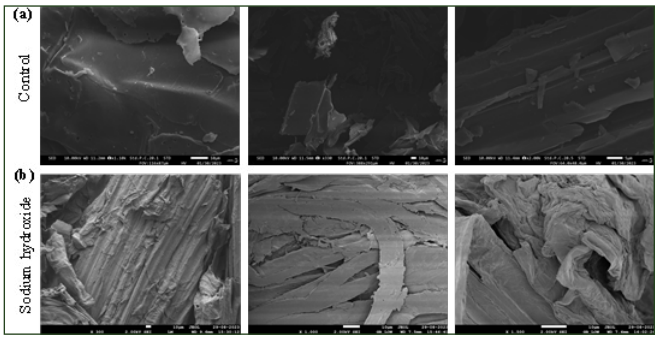

Figure 2: SEM microphotographs depict the surface changes in sugarcane bagasse (a) before (control) and (b) after sodium hydroxide pretreatment

Table 1: Compositional studies of untreated and sodium hydroxide pretreated sugarcane bagasse biomass

Sugarcane bagasse	Untreated Biomass	NaOH (0.3%)	NaOH (0.6%)	NaOH (0.9%)	NaOH (1.2%)
Glucan	37.13	41.23	45.13	50.61	53.81
Xylan	21.89	20.97	19.07	18.14	16.94
AIL	17.17	16.28	15.17	14.11	13.55
ASL	6.59	5.01	4.23	3.79	3.08
Ash	8.10	6.89	5.76	4.87	4.11
OE	9.12	9.62	10.64	8.48	8.51

3.4. Enzymatic hydrolysis of pretreated sugarcane bagasse using trichoderma harzianum

The observed correlation between enzyme dosage and saccharification efficiency in hydrolyzing pretreated sugarcane bagasse with *Trichoderma harzianum* cellulase emphasizes the importance of enzyme concentration in breaking down cellulose and hemicellulose into fermentable sugars. Increased enzyme dosages (5–20 FPU g^{-1}) lead to higher glucose yields, indicating improved substrate accessibility and more efficient hydrolysis (Figure 3). Optimal saccharification efficiency, seen with 15 FPU g^{-1} of substrate releasing 254.43 mg g^{-1} of reducing sugars after 40 h, underscores the critical role of enzyme dosage in maximizing sugar liberation. The sustained increase in reducing sugar release over 40 h suggests persistent enzymatic activity and ongoing polysaccharide deconstruction. Many studies found that once this optimal incubation time was achieved, no further significant enhancement in the release of reducing sugars was observed, suggesting an efficiency plateau in the hydrolysis process (Saini et al., 2023). However, when the hydrolysis time is prolonged, it leads to a decrease in the release of reducing sugars. This decrease can be attributed to the accumulation of sugars, which has the effect of inhibiting the activity of the enzymes involved and, ultimately, reducing the overall yield of the hydrolysis process (Chakraborty et al., 2016; Pathak et al., 2014). These findings underscore *Trichoderma harzianum* cellulase's effectiveness in facilitating efficient saccharification of pretreated SCB, thereby enhancing the potential for biofuel production from lignocellulosic biomass.

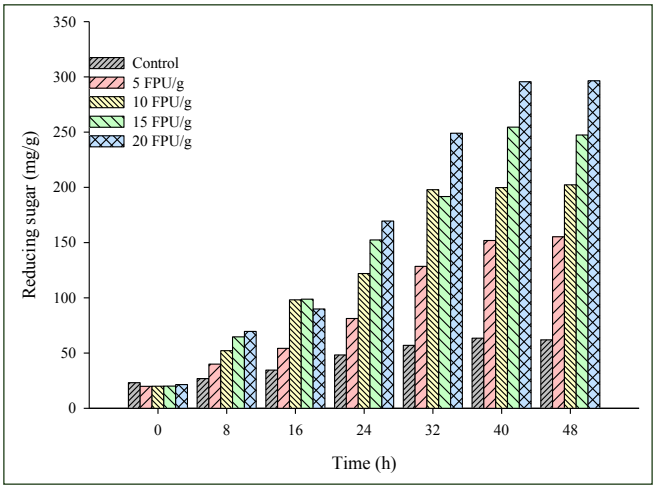

Figure 3: Release of reducing sugars during the enzymatic hydrolysis of alkali pretreated SCB using crude cellulases extracted from *T. harzianum*

3.5. Enzymatic hydrolysis of pretreated sugarcane bagasse using TV

The observed positive correlation between enzymatic dosage and saccharification efficiency in the hydrolysis of pretreated sugarcane bagasse using *Trichoderma viride* cellulase underscores the significance of enzyme concentration in

facilitating the breakdown of cellulose and hemicellulose into fermentable sugars. Increasing enzyme dosages (5-20 FPU g^{-1}) result in higher glucose yields, reflecting enhanced substrate accessibility and more efficient hydrolysis (Figure 4). Optimal saccharification efficiency is achieved at 15 FPU g^{-1}, releasing 325.53 mg g^{-1} of reducing sugars after 40 h, although the maximum sugar release of 350.19 mg g^{-1} occurs with 20 FPU g^{-1} after 48 h. Extended hydrolytic time up to 40 h demonstrates a sustained increase in reducing sugar release, indicating continual enzymatic activity and progressive polysaccharide deconstruction. In more specific terms, the extended hydrolysis time brings about changes in enzyme efficiency due to alterations in the physicochemical characteristics of the substrate and the build-up of end products from the hydrolysis process (Baksi et al., 2023). These inhibitory conditions that arise in the later stages of hydrolysis significantly hamper the performance of the hydrolytic enzymes, resulting in a reduction in the yield of the hydrolysis process (Pathak et al., 2014; Sukumaran et al., 2021). These findings underscored the importance of enzyme concentration in maximizing sugar liberation and highlight the effectiveness of *T. viride* cellulase in facilitating efficient saccharification of pretreated sugarcane bagasse, thereby contributing to the potential for biofuel production from lignocellulosic biomass.

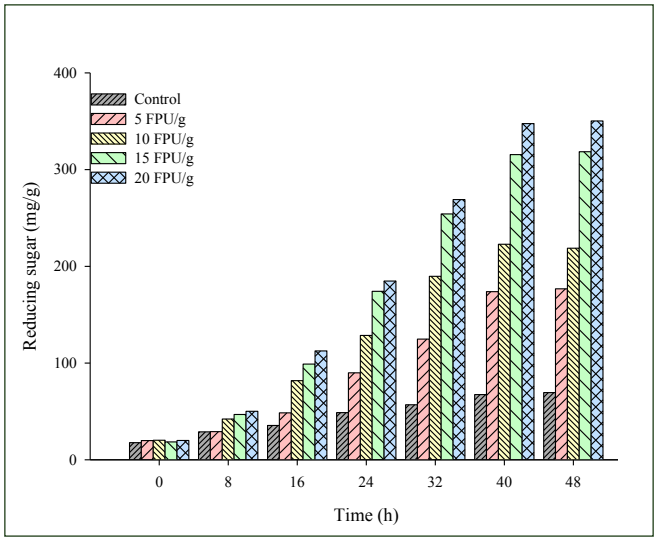

Figure 4: Release of reducing sugars during the enzymatic hydrolysis of Alkali pretreated SCB using crude cellulases extracted from *T. viride*

3.6. Enzymatic hydrolysis of pretreated SCB using enzymatic cocktail

The process of enzymatic saccharification was carried out on pretreated SCB utilising a synergistic enzyme cocktail obtained from *T. viride* and *T. harzianum*. The enzyme cocktail was prepared by mixing crude enzyme extracts from both fungi in a 1:1 volume-to-volume ratio, therefore increasing the saccharification process. The experimental data showcases a clear enhancement in saccharification efficiency across varying dosages of the enzyme cocktail. Particularly, at the highest dosage of 20 FPU g^{-1}, there was a remarkable increase in glucose yields (376.12 mg g^{-1}) compared to the control (72.90 mg g^{-1}) (Figure 5). At optimal dosage 15 FPU g^{-1}, the enzyme cocktail from *T. viride* and *T. harzianum* achieved a substantial increase in glucose yields (345.12 mg g^{-1}) at 40 h, showcasing the synergistic effect of the combined enzymatic activity. This finding not only emphasizes the importance of balanced enzyme compositions but also offers valuable insight into refining enzymatic saccharification strategies for enhanced lignocellulosic biomass conversion (De Oliveira Rodrigues et al., 2022). This synergistic effect of the enzyme cocktail is evident in the improved degradation of cellulose and hemicellulose into fermentable sugars.

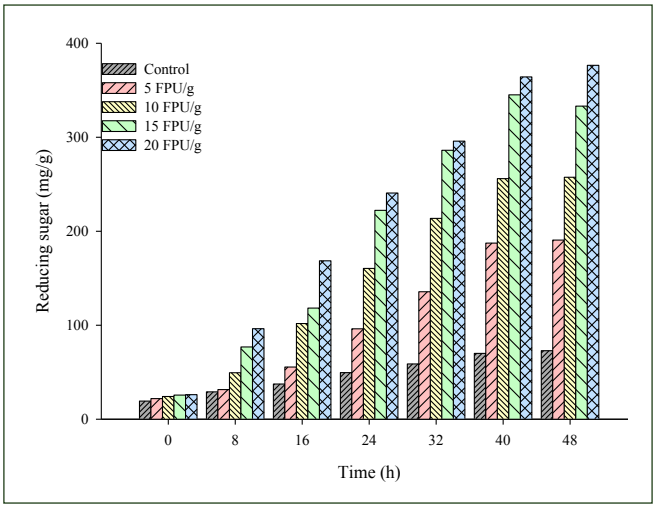

Figure 5: Release of reducing sugars during the enzymatic hydrolysis of Alkali pretreated SCB using crude cellulases extracted from enzymatic cocktail

4. Conclusion

A customized cellulase cocktail from *Trichoderma harzianum* and *Trichoderma viride* enhanced saccharification from alkali-pretreated sugarcane bagasse. This study highlighted synergistic effects of these enzymes in breaking down lignocellulosic structures, boosting fermentable sugar release. By optimizing enzyme dosages and incubation times, significant improvements in saccharification efficiency were achieved. Such research fosters cost-effective, eco-friendly bioconversion, vital for maximizing lignocellulosic biomass's renewable energy potential.

5. Further Research

This study lays the groundwork for future exploration aimed at enhancing enzyme production for more efficient utilization of sugarcane bagasse in ethanol production. Subsequent investigations could concentrate on refining these processes for industrial-scale implementation, thus improving overall efficiency in biofuel production. Moreover, exploring the applicability of these insights to diverse lignocellulosic biomass

sources has the potential to expand the horizon of sustainable bioenergy production.

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