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# Mechanistic insights and parameter optimization for enhanced reducing sugar production from enzymatic hydrolysis of oil palm residues

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## ABSTRACT

Oil palm empty fruit bunches (EFB) and mesocarp fibers (MF) are residual materials from the palm oil industry, distinguished by their notably high levels of cellulose. These lignocellulosic residues serve as valuable raw materials due to their substantial cellulose proportion, making them suitable for various bioconversion processes. In this study, ozonolysis pretreatment was employed to concentrate the cellulose present in EFB and MF, while simultaneously removing lignin content. Subsequently, the cellulose was hydrolyzed enzymatically with cellulase to produce reducing sugars. The contents of cellulose, hemicellulose, and lignin in both raw materials and samples were analyzed through the α-cellulose, γ-cellulose, and Kappa methods, while reducing sugars were quantified using the DNS (3,5-dinitrosalicylic acid) assay. The objective was to evaluate the relative effectiveness of ozonolysis as a pretreatment and to examine how enzyme concentration and reaction duration influence substrate conversion and sugar production from EFB and MF. This analysis provides valuable insight into optimizing the enzymatic hydrolysis process for these biomass materials. Results revealed a progressive increase in cellulose composition for both substrates, with MF showing a more pronounced response than EFB. The reducing sugar content for EFB and MF samples with varying enzyme concentrations increased continuously, reaching an optimum at 42 h. The maximum reducing sugar concentration for EFB was 0.1976 mg/mL, obtained with 15% (w/w) cellulase enzyme (Novozyme), whereas for MF, the highest reducing sugar concentration was 5.23 mg/mL with 10% (w/w) cellulase enzyme treatment.

**Keywords:** empty fruit bunches, mesocarp fibers, ozonolysis pretreatment, enzyme hydrolysis, reducing sugar.

## INTRODUCTION

The worldwide transition to renewable energy has accelerated efforts to identify sustainable raw materials that can effectively sustain large-scale bioenergy generation and biotechnological processes. Among various biomass resources, oil palm stands out due to its significant contribution to the agricultural sector, especially in nations like Indonesia where it significantly supports economic activity and production (Chrisendo et al., 2021; Kurniawan et al., 2025). Growth in palm oil cultivation has resulted in the production of substantial quantities of lignocellulosic residues, especially EFB and MF, which have potential for further exploitation and utilization in bioconversion processes. Although frequently regarded as agricultural residues, these materials offer substantial promise for conversion into valuable carbohydrates, serving as potential substrates for biochemical production processes. This biomass resource thus represents a significant possibility for sustainable material utilization and bio-based product development in related industries (Darus et al., 2022; Suhada et al., 2021; Yasim-Anuar et al., 2017), which serve as essential precursors for bioethanol and other value-added bioproducts. Despite their plentiful availability, effectively

utilizing palm residues continues to be hindered by their inherently resistant and complex structural composition. This resistance limits the ease with which these materials can be processed and converted, thereby posing significant challenges for their use in sustainable production and value-added applications. The intricate composition of structural polymers in EFB dan MF creates a barrier that limits enzyme penetration, causing lower than expected release of sugars during enzymatic breakdown. This structural complexity creates a significant barrier to efficient conversion processes, thereby limiting the overall yield of sugar products from these materials (Brown et al., 2024; Kamdem Tamo et al., 2025). Efficient conversion of these residues into sugar compounds is vital for enhancing the financial viability and environmental sustainability of bioenergy production systems. Enhancing this conversion process can significantly contribute to making bioenergy a more competitive and viable renewable resource (Ojo-kupoluyi et al., 2024; Saravanan et al., 2025). Enzymatic hydrolysis has become a highly promising technique for breaking down complex plant materials, offering benefits such as operating under gentle conditions, high specificity, and generating fewer inhibitory side-products compared to chemical alternatives (Singh et al., 2024; Vinuthana et al., 2025; Wang et al., 2024). This method supports more sustainable and efficient conversion processes. The effectiveness of enzymatic hydrolysis largely hinges on the precise optimization of process parameters, including enzyme concentration and reaction time, which directly influence the extent of substrate conversion and sugar yield.

Previous studies have demonstrated varied degrees of success in the hydrolysis of individual oil palm residues such as empty fruit bunches (Akita et al., 2021; Ghazali and Makhtar, 2018; Sugiwati et al., 2021), fronds (Azani et al., 2024), and trunks (Billateh et al., 2024; Bukhari et al., 2023), yet comprehensive comparative analyses addressing both mechanistic aspects and process optimization remain limited. However, comprehensive comparative studies that integrate mechanistic understanding of substrate conversion with systematic process optimization are still scarce. Recognizing these challenges, the current study endeavors to deliver a detailed comparative assessment of reducing sugar generation from oil palm EFB and MF through enzymatic hydrolysis. The investigation was designed to comprehensively evaluate the comparative efficiency of reducing sugar production from ozonolysis-pretreated EFB and MF through enzymatic hydrolysis. The study additionally aimed to examine the impact of differing enzyme concentrations and reaction times on the effectiveness of substrate conversion and subsequent sugar yield. Comprehending these effects is critical for refining process parameters to enhance both yield and operational efficiency. The amount of reducing sugar generated was measured using the DNS assay, a colorimetric assay that measures the concentration by detecting the intensity of the colored compound formed in the reaction. Furthermore, the study sought to uncover the mechanistic basis for observed differences in hydrolysis performance between these two types of oil palm residues. By pursuing these aims, the work intends to address existing knowledge gaps related to increasing the value and practical use of biomass derived from palm sources. It aims to offer relevant insights that aid in optimizing processing techniques applicable to renewable energy production and biotechnological innovations, thereby advancing sustainable and efficient bioresource applications.

## MATERIALS AND METHODS

The materials employed in this research comprised EFB and MF as the primary substrate, potassium sodium tartrate tetrahydrate (Merck) at a concentration of 40 g/L, sulfuric acid ($H_2SO_4$, Merck) with a concentration of 98%, oxygen gas ($O_2$, medical grade), cellulase enzyme (Novozyme 188, Novozymes) applied at varying concentrations according to experimental design, distilled water (aquadest, Brataco), sodium hydroxide (NaOH, Merck) at 1 N, filter paper (Whatman No. 42), aluminum foil (Merck), DNS reagent (Sigma-Aldrich), citrate buffer solution at pH 4.8 (prepared from citric acid and sodium citrate, Merck), biuret reagent (Merck), and potato dextrose agar (PDA, Merck) for microbial analysis. All substances utilized in this investigation were obtained with certified high purity and applied directly without any further treatment.

### Ozonolysis pretreatment

Each experimental batch consisted of 25 g of EFB and MF powders, which were pre-processed by sieving to ensure a consistent particle size of

60 mesh. The prepared samples were then conditioned by adding distilled water at 10% of the sample weight (v/w) to ensure adequate moisture content for subsequent processing. The prepared samples were subsequently stored at low temperature for 24 hours before undergoing ozone deionization. Samples adjusted to 10% moisture content were then transferred into an ozone reactor, where the ozone flow rate was set at a variable 1 L/min, 2 L/min, and 3 L/min. The ozonation process for the palm fibers was conducted for a duration of 5 min. Both EFB and MF were subjected to filtered ozone treatment, this was done by subsequent treatment with heated water until the samples reached a state of balanced acidity. This washing procedure ensured the removal of residual reactive agents and prepared the samples for subsequent processing and analysis.

## Cellulose analysis

The amount of cellulose was assessed through the α-cellulose technique. Initially, a 1.5 g sample (designated as W) was placed into a 300 mL beaker, followed by the addition of 75 mL of 17.5% NaOH solution, maintained at 25 °C. The sample and alkali solution were thoroughly stirred to ensure complete dispersion while avoiding foam formation. To maintain volume uniformity, the stirring apparatus was washed with an extra 25 mL of NaOH solution, achieving a final volume of 100 mL. After 30 min, 100 mL of purified water was added, and the mixture was stirred before being filtered through a muslin cloth. The initial 10 mL segment of the filtrate was excluded, and the subsequent 100 mL fraction was reserved for subsequent analysis. A 25 mL aliquot was withdrawn and transferred into a 250 mL volumetric flask, followed by the addition of 10 mL of 0.5 N potassium dichromate. Fifty milliliters of concentrated sulfuric acid were slowly introduced into the flask while it was being gently swirled. The mixture was heated at 125 °C for 15 min with boiling chips to prevent bumping. Following heating, 50 mL of purified water was introduced, and the solution was allowed to cool to ambient temperature. Subsequently, a precise volume of ferroin indicator was introduced, and titration was conducted with 0.1 N ferrous ammonium sulfate until the appearance of a violet endpoint. A procedural blank titration was executed identically, substituting the sample filtrate with 12.5 mL each of 17.5% NaOH and purified water. The quantification of α-cellulose was performed using a validated formula incorporating titration volumes (V1 for the blank, V2 for the sample), titrant volume, and normality, as detailed below.

$$Cellulose\ (\%) = \\ = 100 - \frac{6.25\ (V1 - V2)\ \times\ N\ \times\ 20}{A\ \times\ W} \quad (1)$$

## Hemicellulose analysis

The hemicellulose content was assessed using the γ-cellulose assay. A 50 mL portion of the α-cellulose solution was precisely measured and added to a receptacle with a capacity of 100 mL, to which 50 mL of 3N sulfuric acid was added, followed by dilution to volume and thorough homogenization by inversion. The resultant solution was heated at 70–90 °C in a 250 mL Erlenmeyer flask to promote coagulation and subsequently allowed to sediment overnight. After filtration, a 50 mL portion of the clear filtrate was combined with 10 mL of 0.5 N potassium dichromate and 90 mL of concentrated sulfuric acid, added gradually with continuous swirling. The mixture was diluted with 50 mL of purified water, cooled to room temperature, and titrated with 0.1 N ferrous ammonium sulfate in the presence of ferroin indicator until reaching a violet endpoint. A procedural blank was executed under identical conditions. Quantification of γ-cellulose, representing hemicellulose content, was performed using a validated titration-based formula derived from corresponding titrant volumes.

$$Hemicellulose\ (\%) = \\ = \frac{6.85\ (V4 - V3)\ \times\ N\ \times\ 20}{25\ W} \quad (2)$$

## Lignin analysis

Lignin quantification was performed utilizing the kappa number determination method. A precisely weighed one g dry sample (W) was introduced into an Erlenmeyer flask containing 200 mL of deionized water. The flask was placed in a temperature-controlled water bath maintained at 25 °C and subjected to continuous magnetic stirring to ensure uniform reaction conditions. Concurrently, an oxidizing solution comprising 25 mL of 0.1 N potassium permanganate and 25

mL of 4.0 N sulfuric acid was prepared in a 500 mL beaker and subsequently added to the sample-containing flask. The beaker was rinsed with 5 mL of deionized water, which was also transferred to the reaction vessel to maximize reagent recovery. The mixture was allowed to react for 10 minutes, after which 5 mL of 1.0 N potassium iodide was introduced to reduce residual permanganate. The liberated iodine, indicated by a yellow coloration, was immediately titrated with standardized 0.2 N sodium thiosulfate until a violet endpoint was reached. The volume of titrant consumed (a) was recorded. A concurrent blank titration under identical conditions, excluding the sample, yielded volume (b). The lignin content was computed based on these titration volumes following a standardized kappa number calculation protocol.

$$p = \frac{(b - a)\, N}{0.1\, N} \tag{3}$$

$$K = \frac{p \times f}{w} \tag{4}$$

### Enzymatic hydrolysis

Enzymatic hydrolysis was conducted on 10 g of the delignified substrate using cellulase from Novozyme. The enzyme mixture was calibrated to maintain a ratio of 1:15 between endoglucanase and exoglucanase activities throughout the reaction. The substrate was combined with 200 mL of citrate buffer at pH 4.8, and the mixture was stirred until uniform. The pH was verified and adjusted with 10% $H_2SO_4$ as necessary to reach approximately 4.8. Subsequently, reaction vessels were sterilized in an autoclave at 121 °C and 1 atm for 60 min. Cellulase enzyme was added at 100 µL, maintaining a 1:100 ratio based on the 0.05 M acetate buffer volume included in the mixture. The vessels were sealed with cotton and aluminum foil. Hydrolysis was performed at 40 °C for various time intervals from 6 to 48 hours. Samples (1 mL) were withdrawn at each interval and analyzed for glucose concentration via spectrophotometry.

### Glucose analysis

The quantification of glucose was performed using the DNS assay, a well-established colorimetric method that detects reducing sugars. In this protocol, equal volumes of the test sample or glucose standard and DNS reagent were mixed and heated at 100 °C for 5 min to facilitate the formation of a colored complex. The reaction mixture was then rapidly cooled to stop the process. To stabilize the color, sodium potassium tartrate solution (40%) was added. Absorbance readings were taken at 540 nm using a spectrophotometer, with glucose concentrations deduced by comparing absorbance values against a standard calibration curve prepared from known glucose levels.

## RESULT AND DISCUSSION

### The effect of ozone flowrate on lignocellulose composition

The lignocellulose composition of EFB and MF raw materials were analyzed both prior to and following the delignification process. The compositional data for cellulose, hemicellulose, and lignin are presented in Figure 1 (EFB) and Figure 2 (MF). As illustrated in Figures 1 and 2, variations in ozone flow rate exert a direct influence on gas-liquid-solid contact, mass transfer coefficients, and ozone distribution within the biomass matrix, ultimately affecting delignification efficiency and the relative composition of each lignocellulosic component. Incremental increases in ozone flow rate induce distinct compositional trends for each substrate. The mass transfer of ozone into the biomass matrix is governed by the gas flow rate, whereby higher flow rates enhance turbulence and interfacial contact among the gas, liquid, and solid phases (Harahap et al., 2019).

The compositional changes in cellulose exhibited a progressive increase in both substrates, with MF demonstrating a more pronounced response compared to EFB. This phenomenon suggests that MF possessed a more responsive morphological structure to ozonolysis, likely attributable to differences in porosity, particle size, and lignin distribution within the cellulose matrix (Nabila et al., 2023; Pereira et al., 2020; Suhada et al., 2021). The observed rise in cellulose content does not reflect an absolute increase in cellulose quantity. Rather, it stems from the preferential degradation of lignin and hemicellulose, this leads to a higher proportion of cellulose present within the structural framework of the biomass. (Chen et al., 2022; Gao et al., 2025). Hemicellulose reduction within EFB occurred at a measured rate, displaying only slight variation at lower ozone

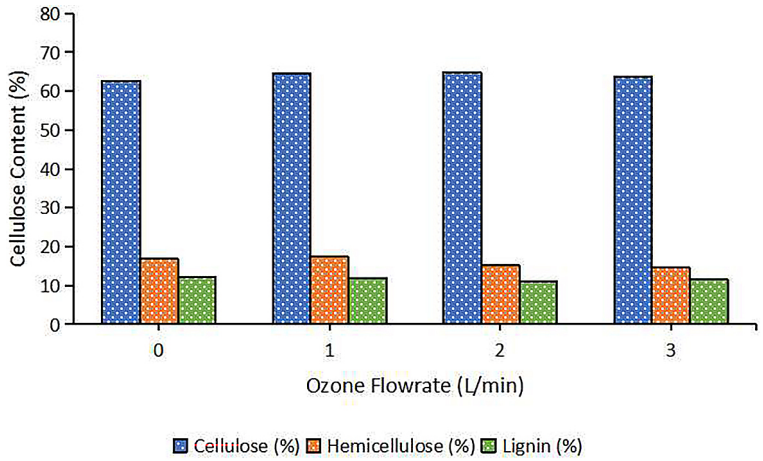


**Figure 1.** Lignocellulose composition of EFB

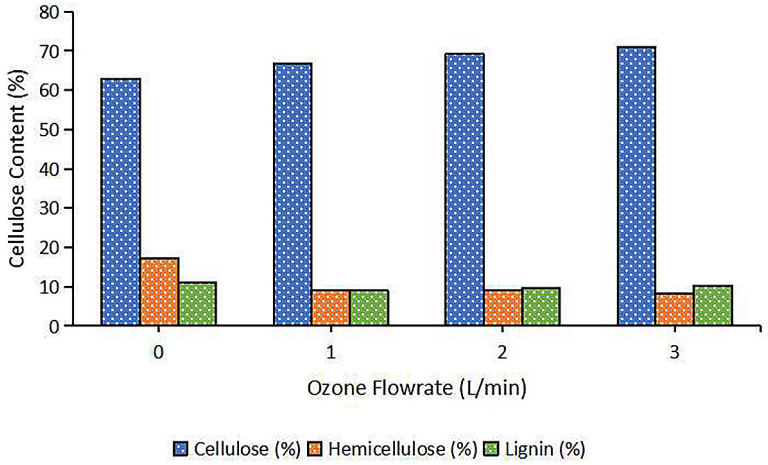


**Figure 2.** Lignocellulose composition of MF

flow rates and ultimately reaching a peak loss of 14.08% when the flow rate was increased to 3 L/min. This outcome implies that the hemicellulose fraction in EFB is comparatively more resistant to oxidative degradation by ozone (Mahardika et al., 2024), which may be attributed to its elevated crystallinity or the structural protection afforded by a densely packed lignin matrix. In contrast, MF exhibited a much greater extent of hemicellulose depletion. The heightened susceptibility of hemicellulose in MF is largely attributable to the action of ozone, which cleaves β-1,4 glycosidic bonds within the hemicellulose structure (Jannah et al., 2024). This branched configuration renders hemicellulose more accessible to oxidative attack compared to the more robust β-1,4-glucosidic linkages characteristic of cellulose.

In this study, the effectiveness of ozonolysis as a pretreatment was evaluated primarily through the extent of lignin removal from each biomass sample (EFB and MF). Lignin is known to act as a physical and chemical barrier that limits enzyme access to cellulose and hemicellulose; therefore, its removal or redistribution is a key objective of pretreatment. A higher degree of lignin removal generally indicates a more effective disruption of the lignin–carbohydrate matrix and improved substrate accessibility, which in turn is expected to facilitate enzymatic hydrolysis and increase reducing sugar yields.

The ozonolysis process in lignocellulosic biomass involves a complex series of oxidative reactions, starting with ozone targeting sites within the lignin structure that possess high electron density. This interaction initiates the breakdown of lignin, facilitating further depolymerization and modification of the biomass components (Dey et al., 2025; Mujtaba et al., 2023; Zhou et al., 2023). As a highly potent oxidant ($E° = 2.07$ V), ozone preferentially reacts with carbon–carbon double

bonds in lignin side chains, aromatic centers of syringyl and guaiacyl units, and the predominant β-O-4 linkages in the lignin polymer. The flow rate of ozone directly influences both the distribution and concentration of dissolved ozone in the liquid phase, thereby modulating the kinetics of the oxidation reactions. At lower flow rates, the concentration of dissolved ozone tends to be higher, although its dispersion within the biomass matrix may be less uniform. Conversely, higher flow rates enhance ozone distribution throughout the matrix, but may result in lower local concentrations due to reduced contact time.

Ozonolysis is generally considered a lignin-selective pretreatment; however, several potential side effects must be taken into account. Under severe or prolonged ozonation, cellulose may undergo partial depolymerization and oxidative cleavage, leading to a decrease in its degree of polymerization and, in extreme cases, a loss of cellulose available for subsequent enzymatic hydrolysis. In addition, residual lignin can non-productively adsorb cellulolytic enzymes through hydrophobic and electrostatic interactions, thereby lowering the effective enzyme concentration in the liquid phase and reducing hydrolysis efficiency. Moreover, reactive oxygen species generated during ozonolysis may also oxidize soluble sugars released from the biomass, forming carbonyl or acidic derivatives and potentially decreasing the measurable reducing sugar concentration. These phenomena together can influence the apparent effectiveness of ozonolysis and must be considered when interpreting the hydrolysis results.

## The effect of enzyme concentration and reaction time on the hydrolysis

The hydrolysis of EFB dan MF involved the cellulase enzyme (Novozyme). The enzyme's catalytic performance in batch hydrolysis is highly dependent on the enzyme concentration used and the duration of the hydrolysis process. The analysis of reducing sugar content was conducted using a spectrophotometric method by first preparing a glucose standard solution, the results of which were then plotted as a graph. The reducing sugar concentration obtained from the hydrolysis is presented in Figure 3.

Based on Figure 3, the reducing sugar content for EFB dan MF samples with varying enzyme concentrations continuously increased and reached an optimum at 42 h. The maximum reducing sugar concentration for EFB was obtained in the sample treated with 15% (w/w) cellulase enzyme (Novozyme), measuring 0.1976 mg/mL. Meanwhile, for MF, the highest reducing sugar concentration was observed in the sample treated with 10% (w/w) cellulase enzyme, reaching 5.23 mg/mL. Enzyme activity was highly pronounced after 42 h of hydrolysis, reflecting that the cellulase enzymes were nearing the completion of the exponential phase and entering the stationary phase. During the exponential phase, the microorganisms producing cellulase enzymes begin to divide at an increasing rate, resulting in a significant increase in enzyme production (Borthakur et al., 2024). Extensive interaction between the enzyme and substrate leads to the production of a significant amount of reducing sugars. The elevated activity of the cellulase enzyme plays a crucial role in achieving the highest reducing sugar yield within a 42-h period (Dewiyanti et al., 2021). Beyond 42 h of hydrolysis, reducing sugar production begins to decline. By 48 hours, the cellulase enzyme's effectiveness is reduced due to the depletion of substrate availability. Furthermore, at the 42-h mark, the cellulase enzyme transitions from the stationary phase into the death phase, during which its activity sharply decreases. Enzymes that become inactive can no longer interact with the cellulose substrate, resulting in the cessation of the hydrolysis process (Kurniawati et al., 2019). Extended hydrolysis time leads to increased formation of by-products such as cellobiose and dextrin. These compounds can disrupt the hydrolysis process and decrease the overall efficiency of glucose production.

The concentration of cellulase enzyme in EFB dan MF significantly influenced the reducing sugars. The highest enzyme involvement in EFB samples resulted in the maximum reducing sugars. However, for MF, the optimum time to achieve maximum reducing sugar varied with different cellulase enzyme concentrations. The variation in optimal reducing sugar production time on MF may result from a combination of factors such as enzyme availability, hydrolysis efficiency, by-product accumulation, and environmental conditions. Low enzyme concentration (5% (w/w) was insufficient to efficiently break down the substrate within a short time, causing reducing sugar production to increase but reach its peak faster due to enzyme limitations. The maximum concentration of reducing sugars produced by

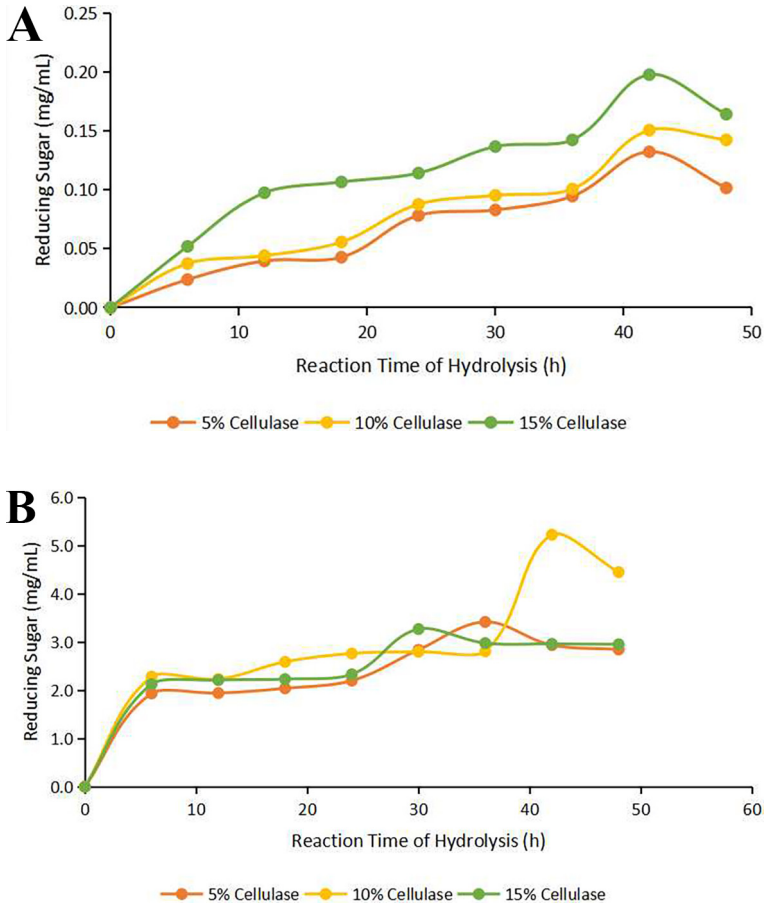


**Figure 3.** Reducing Sugar of (a) EFB (b) MF

samples with a 5% (w/w) enzyme concentration was only 3.42 mg/mL. This value was significantly lower than the maximum reducing sugar yield of 5.23 mg/mL obtained from samples treated with a 10% enzyme concentration. Higher enzyme concentrations corresponded proportionally to longer hydrolysis times needed to reach optimal conditions. However, in samples with a 15% (w/w) enzyme concentration, the reducing sugar yield was lower compared to those with a 10% concentration, indicating that an excessively high cellulase enzyme concentration resulted in inefficient substrate conversion to reducing sugars.

The reducing sugar concentration produced by MF was significantly higher compared to EFB. The disparity was attributed to the higher cellulose content in MF following delignification compared to EFB, along with a lower lignin concentration. Variations in lignocellulosic composition were the primary factors influencing the hydrolysis process. Accessibility to cellulose and hydrolysis yield improve as the amount of delignified lignin increases, since lignin adsorbs onto cellulose and decreases the surface area accessible to enzymes. (Chan et al., 2021). The elimination of lignin during delignification enhanced the hydrolytic performance of cellulose by enzymes, as cellulase adhered to lignin through interactions involving hydrogen bonds and other intermolecular forces. Reducing lignin presence decreases these adhesive interactions, thereby facilitating greater enzyme access to cellulose and promoting more effective breakdown. This binding obstructed the enzyme sites responsible for specific substrate interaction (Liu et al., 2016). The prevention of cellulase adsorption by lignin can be achieved through the addition of additives such as bovine serum albumin (Chan et al., 2021). Hydrophobic interactions occur between the nonpolar regions of molecules. These interactions contribute to substrate structure stabilization by promoting the aggregation of nonpolar segments internally, away from the aqueous environment, thereby reducing interactions between nonpolar residues and water and increasing the system's entropy (Arwansyah et al., 2014; Muhammad et al., 2025; Xiao et al., 2020). Lignin removal during the delignification

process allowed greater access for cellulase enzymes to interact with cellulose, which had previously been obstructed by lignin through hydrophobic interactions (Li et al., 2019).

## CONCLUSION

The practical significance of these results is essential for enhancing the pretreatment process of oil palm biomass. The findings demonstrated a steady enhancement in cellulose content across both substrates, with MF exhibiting a more significant increase compared to EFB. An optimal flow rate of 2 L/min for EFB was identified as providing the most effective balance, resulting in 10.19% delignification combined with a 3.34% increase in cellulose content. In comparison, MF demonstrated a maximum delignification of 19.06% at a lower flow rate of 1 L/min, while the highest cellulose enrichment of 12.78% was achieved at an increased flow rate of 3 L/min. These results highlight the importance of adjusting processing conditions specific to each substrate to optimize both lignin removal and cellulose improvement during biomass pretreatment. However, the reducing sugar content for EFB and MF samples with varying enzyme concentrations increased continuously, reaching an optimum at 42 h. The highest reducing sugar concentration recorded for EFB was 0.1976 mg/mL, which was obtained using a 15% (w/ w) dosage of the cellulase enzyme from Novozyme. In contrast, MF reached its maximum reducing sugar concentration of 5.23 mg/mL when treated with 10% (w/w) cellulase enzyme. This indicates that optimal enzymatic hydrolysis conditions differ between these two substrates, likely due to their distinct structural and compositional characteristics that influence enzyme effectiveness over time.

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