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Improving the enzymatic hydrolysis of rubberwood sawdust wastes pretreated by microwave-assisted alkaline pretreatment

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ABSTRACT

Rubberwood sawdust comprises cellulose, serving as a feedstock for bioethanol production. Cellulose from pretreated rubberwood was transformed to reducing sugars by enzymatic hydrolysis via a crude enzyme sourced originate from *Aspergillus niger*. This research aims to clarify the kinetics of enzymatic hydrolysis for durations of 5, 10, 15, 20, and 25 hours at enzyme-to-substrate ratios of 1:20, 2:20, 3:20, 4:20, 5:20, and 6:20 (v/w). The results showed that, at an enzyme-to-substrate ratio of 6:20 (v/w) for 25 h, the maximum quantity of reducing sugars was approximately 3.15 g L^{-1}. The FPase activity was 255.1672 U mL^{-1}, whereas the CMCase activity reached 235.9105. The Michaelis-Menten kinetic model states that the maximum rate ranges from 6.50×10^{-7} to 2.6×10^{-6} Mol L^{-1} s^{-1}, and the reaction rate produces a Michaelis constant between 0.0051 and 0.0055.

Keywords: enzymatic hydrolysis kinetics, Michaelis-Menten model, microwave-assisted alkaline pretreatment, rubberwood sawdust, reducing sugar.

INTRODUCTION

With the rise of coal, crude oil, and natural gas in the 18th and 19th centuries, fossil energy represents a period of societal change that led to improved production, efficiency, transportation, and standard living methods (Lee and Yang, 2019). The reliance on fossil fuels is hindered by their fast depletion, limited availability, significant greenhouse gas emissions, global warming, and other ecological problems that threaten the existence of the Earth (Mankar et al., 2021). Consequently, each country must swiftly shift from fossil fuels to alternative energy resources and chemicals composed of carbon to guarantee an environmentally friendly carbon society. In this sense, research on reliable, sustainable, and renewable alternatives to fossil fuels has attracted attention from the government, industry, and academia in the twenty-first century. It is increasingly acknowledged that photosynthetic carbon stored in plant biomass offers a copious supply of clean, sustainable, and ecologically friendly carbon for the synthesis of chemicals, fuels, and other valuable materials (Liao et al., 2020). The commercialization of energy compounds from biomass has been the focus of significant corporate and government initiatives in recent years. The worldwide scientific community, however, criticized the initial manufacturing models since they focused on consumable sugar and starch commodities or oils as raw materials (Arhin et al., 2023). The most abundant type of biomass is lignocellulosic, which includes yard trash, crops, and woody residues, mostly cellulose, hemicellulose, and lignin (Deng et al., 2023). Only 8.2 billion of the 200 billion tons of lignocellulosic biomass produced each year worldwide are used for material, energy, and agricultural purposes (Arhin et al., 2023).

Lignocellulose is a component of agricultural material that may be converted into various products, including bioethanol, biogas, bio-oil, charcoal, and nanocellulose, which are used in the electronics, industrial, and transportation sectors.

Rubber trees (*Hevea brasiliensis*) are Indonesia's primary and dependable agricultural commodity. The Ministry of Agriculture of the Republic of Indonesia (2022) reported that rubber cultivation in Indonesia exceeded 3.7 million hectares by 2022. The plant region is mostly located in six regions, contributing 73.86%: south Sumatra, west Kalimantan, Jambi, north Sumatra, and central Kalimantan. Rubber plants possess a commercial lifespan of 25 to 30 years prior to rejuvenating. Rubber plants may provide wood after their harvesting phase, representing a prospective bioenergy supply with a relatively large lignocellulose content.

Harahap et al. (2022) assert that preliminary pretreatment is necessary to improve cellulase enzyme availability of cellulose and reduce the formation of inhibiting enzymes throughout the process. Lignocellulose pretreatment may be conducted using combined physical and chemical methods, namely alkali pretreatment using potassium hydroxide (KOH) and microwave heating. By changing the crystal structure of cellulose types and removing parts of lignin and hemicellulose, alkali pretreatment is a chemical technique that modifies the structural integrity of lignocellulosic materials (Novia et al., 2024). Alkali pretreatment is an effective method for eliminating lignin constituents and deconstructing biomass composition. Alkali pretreatment is comparatively economical due to its moderate reactions, recoverability, and good selectivity for lignin extraction (Kim et al., 2016). Because it may break down the lignocellulosic matrix and eliminate acetyl groups, KOH is a useful pretreatment chemical. This combination functions as a potent alkali and is more ecologically friendly than NaOH and $Ca(OH)_2$ (Bensah et al., 2019) and supplying wastewater with potassium (K) molecules to support plant growth (Li et al., 2015). Zahoor et al. (2021) showed that KOH pretreatment (KP) enhanced the generation of sugar yield from maize stovers, and the resulting liquid had a favorable effect on plant development. Therefore, employing KOH is more economical and environmentally favorable than developing a sustainable bioethanol manufacturing process.

Heating is generally needed to help the reaction go faster. However, conventional heating tends to be challenging in controlling temperature and time, so it needs to be assisted by wave irradiation, one of which is microwave. Because of its quick treatment duration, selective processing, immediate control, and faster reaction rate, microwave pretreatment technology has lately been employed to turn lignocellulosic biomass into sugars and ethanol. A potential method utilizing both thermal and non-thermal effects generated by microwaves in aquatic settings is the use of microwave radiation in pretreatment (Harahap et al., 2019). The direct transfer of microwave energy to polysaccharides through molecular interactions with electromagnetic fields may be the reason why microwave radiation is preferable for activating polysaccharides. The polar molecules' vibration and ions' movement generate extensive heat and collisions. Microwave irradiation-based heating shows selective and uniform heating throughout the material with a short reaction time, causing faster disintegration of lignocellulose with fewer by-products (Ho et al., 2020). Non-ionizing electromagnetic radiation is what microwaves are. By causing implosion inside a material's particles, microwave radiation makes it easier to break through resistant formations. Microwaves are electromagnetic energy waves that have frequencies and wavelengths between 0.3 GHz and 300 GHz and 1 m and 1 mm, respectively. Microwave irradiation pretreatment of lignocellulose results in swelling of the fibers, resulting in a higher surface area. Several industries have benefited from microwave heating over conventional heating. Microwave irradiation energy causes volumetric heating of the material rather than just through the surface of the material. Temperature, frequency, and microwave power density are other important variables that affect how well microwave heating is absorbed (Arpia et al., 2021). The microwave power intensity regulates the processing temperature of biomass resources. The temperature and specific energy input rise as the power intensity increases. In this sense, boosting microwave absorption enhances the interaction between materials and microwaves.

Long-chain cellulose, starch, protein, and fat molecules are broken down by a process called hydrolysis into smaller, fermentable molecules like simple sugars, fatty acids, and amino acids. In order to convert cellulose into fuel, this procedure often requires enzymatic hydrolysis to break it down into five or six-carbon sugars (Ananthaswamy et al., 2020). Biomass pretreatment followed by enzymatic hydrolysis has been regarded as a practical method of obtaining sugars from biomass due to its fractionation effect and the great selectivity of enzymes for hydrolyzing polysaccharides (cellulose and hemicellulose) to sugars (such as glucose and xylose) (Luo et al., 2019). Microbial growth in the fermentation process can be described quantitatively by studying kinetics. The Michaelis-Menten constant and the maximal response rate may estimate the kinetics of cellulose growth. The correlation between substrate concentration and cellulose synthesis rate is established by the Michaelis-Menten constant (KM). As the concentration of the substrate rises, so will the rate of cellulose growth. The production rate decreases with decreasing substrate concentration. With an increase in substrate concentration, the rate of cellulose synthesis will rise. This study aimed to determine the optimum sugar concentration for production, maximum speed value (V_{max}), and K_M of the microbial cellulose produced.

The kinetics of the enzymatic hydrolysis of plant tissue, especially banana stem (Novia et al., 2024), rice husks (Novia et al., 2024), and corn cob (Novia, Aji, et al., 2025) have been studied. Nonetheless, no documented studies on the enzymatic hydrolysis of rubberwood sawdust made using microwave-assisted KOH using the Michaelis-Menten model. The goal of this work is to use a semi-mechanistic model to clarify the kinetics of the enzymatic hydrolysis of cellulose made from rubberwood flour. The kinetics of the enzymatic hydrolysis of cellulose made from rubberwood sawdust were examined using the unified Michaelis-Menten formula. Modifications to analytical, theoretical, and empirical data confirmed that the mathematical framework was adequate.

MATERIALS AND METHODS

Biomass and chemicals

Rubberwood sawdust samples were taken from a fibreboard company in Indralaya, Ogan Ilir, south Sumatra. Merck (Indonesia) supplied the chemicals needed for hydrolysis and pretreatment. The source of *Aspergillus niger* was Microbial March Gallery in Indonesia. The cultures were regularly preserved on PDA plates at 30 °C for 4–6 days. The ultimate concentration of the generated spores was 1×10^6 spores mL^{-1} after they were suspended in deionized water.

Preparation of rubberwood sawdust material

For four days, the rubberwood sawdust was dehydrated at 50 °C in an oven. A grinder was then used to crush the sawdust into a fine powder. After that, it was sieved to produce particles with a 20-mesh size. After that, it was preserved and subjected to the Chesson analytical method to determine its cellulose, hemicellulose, and lignin content (Datta, 1981).

Microwave-assisted KOH pretreatment

The prepared rubber wood powder was put into an Erlenmeyer, and then a 5% KOH solution with a 1:10 biomass ratio was put into the Erlenmeyer. After that, the mixture was heated with a hotplate stirrer at 70–80 °C and then put into a microwave at 360 °C for 25 min. Furthermore, the mixture was neutralized with distilled water, filtered, and dried in an oven at 100–110 °C. The Chesson, XRD, and FTIR methods analyzed the samples.

Production of cellulase enzyme from *Aspergillus niger*

Inoculum preparation was carried out with 100 mL of liquid media, consisting of 12.5% sucrose, 0.25% $(NH_4)_2SO_4$, and 0.2% KH_2PO_4, with the pH of the liquid regulated to 3 with the addition of citric acid. The apparatus and liquid media were sterilized in an autoclave at 121 °C for 15 min. With an inoculation loop, the inoculum was made by transporting isolates from PDA media to liquid media aseptically, then incubated for 24 h at around 30 °C in an incubator. Additionally, 20 g of rubber wood powder that had been pretreated with extra nutrients 0.03 g urea, 0.005 g $MgSO_4.7H_2O$, and 0.0023 g KH_2PO_4 as well as 80 mL of deionized water (pH 5) were used to prepare the enzyme growth media for the production of cellulose enzymes. 10 mL of the inoculum was added to the enzyme growth medium, which was then kept at 30 °C for 96 hours. After filtering the filtrate with 100 milliliters of deionized water, it was stirred for one hour at 150 rpm. Additionally, the mixture was centrifuged for 30 minutes at 4 °C and 4000 rpm. The enzymatic hydrolysis was carried out using the obtained crude enzyme. With a UV-Vis spectrophotometer, DNS liquid measured the activity of cellulase enzymes. Using a Bradford solution, the enzyme's protein level was investigated.

Enzymatic hydrolysis

The equipment was autoclaved for 60 minutes at 121 °C to pasteurize it. Rubber wood powder and crude *Aspergillus niger* enzymes were added to an Erlenmeyer with varying ratios of 1:20, 2:20, 3:20, 4:20, 5:20, and 6:20 (v/w) (enzyme fraction in ml/biomass weight in g). At pH 5, the ratio of rubber wood powder to deionization was 1:10 (w/v), controlled by sodium citrate buffer. For hydrolysis periods of 5, 10, 15, 20, and 25 hours, respectively, the samples were warmed at 50 °C and agitated at 200 RPM. Additionally, after cooling the filter, DNS liquid was used to measure the amount of sugar using a UV-Vis spectrophotometer.

Analytical methods

The structure of rubberwood sawdust (cellulose, hemicellulose, lignin) during pretreatment was analyzed by the Chesson technique (Datta, 1981). The elemental structure of untreated and pre-treated rubberwood sawdust was tested using Axia ChemiSem SEM-EDX, FTIR, and XRD.

Chesson method analysis

150 ml of water and 1 g of dry rubberwood sample are refluxed for one hour at 100 °C. Furthermore, hot water was used to filter and cleanse the refluxed solution. 150 mL of 1 N was inserted, the mixture was refluxed, and the residue was weighed (b). After neutralizing the solution to a pH of neutral, the residue was weighed and dried (c). After adding 10 mL of 72% H_2SO_4 and letting it soak for 4 hours at room temperature, 150 mL of 1 N H_2SO_4 was inserted, and the mixture was refluxed for 1 hour at 100 °C. After neutralizing the refluxed solution to neutral, it was dried and balanced (d). Furthermore, the solid (d) was cleaned before being weighed. The sample's lignin, cellulose, and hemicellulose contents (%) were then determined.

$$\text{Hemicellulose content} = (b-c)/a \times 100\% \qquad (1)$$

$$\text{Cellulose content} = (c-d)/a \times 100\% \qquad (2)$$

$$\text{Lignin content} = (d-e)/a \times 100\% \qquad (3)$$

SEM-EDS analysis

Using the JEOL SEM-EDS of JED-2200 SERIES at 15 KV, the morphology of untreated and processed rubber wood sawdust was examined using the SEM test.

X-ray diffraction (XRD) analysis

XRD images were obtained using the Rigaku miniflex Japan diffractometer. The device was run at 15 mA of current and 40 KV of acceleration voltage.

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of the samples were acquired by Fisher's Nicolet 370 FT-IR, with an attenuated total reflection (ATR) assembly module. This was carried out in an absorbance style from 4000 to 500 cm^{-1} at a resolution of 2 cm^{-1}, with around 64 scans, and the whole spectrum required zero amendment.

Cellulase enzyme activity (estimation of endoglucanase activity)

A citrate buffer (pH 4.8) was mixed with 0.1 mL of cellulase enzyme filtrate and 0.1 mL of 1% CMC solution, and the mixture was incubated for 30 minutes at 50 °C. After adding 3 mL of DNS, the mixture was heated in hot water at 100 °C for 10 minutes to finish the process. The absorbance at 540 nm was measured once the temperature reached the ambient level. A glucose standard curve was used to illustrate the absorbance results. The absorbance baseline, which was formed by the citrate buffer and DNS reaction, was evaluated, and blanks were created using these reactions. Based on the amount of glucose released by the cellulase enzyme, one unit of CMCase enzyme activity is equivalent to one mole of glucose produced per minute. At 235.9105 U mL^{-1}, the CMCase activity was measured.

Cellulase enzyme activity (estimation of exoglucanase activity)

Following the addition of about 0.1 mL of cellulase enzyme filtrate, a specimen tube containing 1 × 6 cm of Whatman No. 1 filter paper (50 mg) and 1 mL of citrate buffer at pH 4.8 was incubated for 30 minutes at 50 °C. To stop the reaction, 3 mL of DNS solution was added, and the mixture was boiled in boiling water for 10 minutes. Furthermore, after cooling, the absorbance was determined at a wavelength of 540 nm. The level of glucose generated per minute was applied to measure the FPase enzyme's activity. The activity of FPase reached 255.1672 U mL^{-1}.

Total reducing sugars

Using a UV-Vis spectrophotometer to assess the samples' absorbance, the DNS procedure was utilized to determine the total reducing sugars. One milliliter of the hydrolysis liquid was moved into a tube, mixed with three milliliters of DNS reagent, and heated for five minutes at 100 degrees Celsius in a water bath. After cooling, the solution was put into a cuvette, where absorbance at 540 nm was measured using a UV-Vis spectrophotometer. All experiments in this study were carried out in triplicate, and Microsoft Excel was used to calculate the average deviance.

Enzymatic hydrolysis kinetics

An equation that links the rate of an enzyme reaction to the concentration of the substrate is known as the Michaelis-Menten constant. The enzymatic response in this equation is a reversible process in which the enzyme (E) releases the product (P) and the unchanged free enzyme after binding to the substrate (S) to form a complex (ES). The Michaelis-Menten equation can be written as follows:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} E + P \qquad (4)$$

The Michaelis-Menten enzyme catalyst model (M-M) delineates the reversible interaction between enzymes [E] and substrates [S] to create Enzyme-Substrate [ES] complexes, as well as the irreversible transformation of ES complexes into products [P] and free enzymes. The rate equation associated with the reaction mechanism (Nnaemeka et al., 2021) is:

$$\frac{d[s]}{dt} = -k_1\,[S][E] + k_{-1}\,[E][S] \qquad (5)$$

$$\frac{d[E]}{dt} = -k_1\,[S][E] + k_{-1}\,[E][S] + k_2\,[E][S] \qquad (6)$$

$$\frac{d[ES]}{dt} = k_1\,[S]([E_0 - ES]) - (k_{-1} + k_2)[ES] \qquad (7)$$

$$\frac{d[P]}{dt} = k_2\,[ES] = v \qquad (8)$$

In this context, v denotes the rate of product output, whereas the concentration of the enzyme-substrate complex, ES, is contingent upon the reactant concentrations.

$$\frac{d[ES]}{dt} = \frac{d[E]}{dt} = 0 \qquad (9)$$

$$k_1\,[S]\big[[E_0 - ES])\big] = (k_{-1} + k_2)[E][S] \qquad (10)$$

$$v = \frac{d[P]}{dt} = k_2\,[ES] = \frac{k_2\,[S][E]0}{[s] + \frac{k_{-1} + k_2}{k_1}} \qquad (11)$$

In this equation, v represents the response rate, whereas V_M denotes the maximum rate. The Michaelis-Menten equation and the double reciprocal plot approach determine the initial rate of sugar production.

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad (12)$$

$$V_M = k_2[E]o \qquad (13)$$

$$v = -\frac{d[s]}{dt} = \frac{V_M[S]}{K_M + [S]} \qquad (14)$$

Integration of Equations 11–14 yields

$$K_M \ln\frac{[S]_0}{[S]} + [S]_0 - [S] = V_M.t \qquad (15)$$

$$\frac{[S]_0 - [S]}{\ln\frac{[S]0}{[S]}} = -K_M + k_2[E]o\,\frac{t}{\ln\frac{[S]0}{[S]}} \qquad (16)$$

where:

$$[P] = [S]_0 - [S] \qquad (17)$$

[P]∞ is the concentration of the reaction's final product at t = ∞, whereas [P] is comparable to the product's concentration at time t. It is assumed that [S]0 equals [P]∞ at the conclusion of the reaction.

$$\frac{[P]}{\ln\frac{[P]\infty}{[P]\infty - [P]}} = -K_M + k_2.E_0.\frac{[t]}{\ln\frac{[P]\infty}{[P]\infty - [P]}} \qquad (18)$$

RESULTS AND DISCUSSION

Effect of microwave-assisted alkaline pretreatment on the composition of rubberwood sawdust

Table 1 displays the formation of rubber wood sawdust before and after pretreatment. The percentage component of the variables derived for every pretreatment demonstrates a considerable correlation. For instance, pretreatment using potassium hydroxide decreased the lignin level from 29.83% to 16.13%. The cellulose content in the rubber wood sawdust rose from 35.00% to 55.26%. The decrease in the amount of lignin prevents the inhibition of enzymatic hydrolysis. Consequently, it facilitates the enzymatic breakdown of cellulose into glucose with greater efficiency. A prior investigation (Efrinalia et al., 2022) indicates that decreased lignin concentration in substrates might enhance enzyme effectiveness and activity of microbes, hence decreasing enzyme requirements. An alkali solution functions as a chemical bond-breaking substance, destabilizing the lignin framework in amorphous crystals and isolating certain hemicellulose components.

These changes facilitate changes in structure and increase the solubility of lignin in water (Anggriani et al., 2023). Then, the hemicellulose content decreased from 22.00% to 16.13%. The hemicellulose content also decreased during delignification because the degree of polymerization was small, so it was easily degraded.

Table 1 indicates that the SEM-EDS investigation verified the existence of several elements, including carbon, oxygen, and magnesium. Table 2 indicates a rise in the quantity of carbon atoms after pretreatment with KOH. This occurrence signifies an augmentation in biomass cellulose concentration. Conversely, the quantity of O atoms decreased after pretreatment with KOH. The findings align with previous investigations (Efrinalia et al., 2022; Wati et al., 2025).

SEM-EDS analysis of untreated and pretreated rubberwood sawdust

Morphological structures in lignocellulosic biomass were analyzed to assess lignin removal and its appropriateness for enzymatic hydrolysis after pretreatment. The carbon percentage in rubber wood sawdust rose from 47.4% to 48.4% after pretreatment, as seen in Table 2. The reported rise signifies that the pretreatment phase augmented the cellulose portion while concurrently decreasing the ash (magnesium) level (Novia et al., 2025). The amount of carbon in untreated and treated rubber wood sawdust types exhibited remarkable stability, showing no significant changes. The percentage of ash (magnesium) was decreased, resulting in the elimination of amounts of ash throughout the implementation of pre- and post-pretreatment processes (Moreira et al., 2021). Both approaches influenced the carbon (C) content of rubberwood powder, resulting in a 1% increase. A 0.2% drop in the Mg part of the compound was detected.

Table 2 shows that the carbon value was 47.4%, and after pretreatment, it was 48.4%, which aligns with the research (Efrinalia et al., 2022). The oxygen value from Table 2 also shows a value of 58.1% to 57.5%, which aligns with research (Novia et al., 2025). In the microwave-assisted pretreatment, the magnesium content was reduced from 0,4 %wt to 0,2 % wt. This level decrease occurs due to the pretreatment process in line with the reference (Wati et al., 2025). The structure and composition of the plant cell wall have a significant impact on the effectiveness of enzymatic hydrolysis, a crucial procedure for converting lignocellulosic biomass into fermentable sugars for the production of ethanol (Visković et al., 2024). Plant cell walls are complex molecular structures made up of phenolic components (lignin and p-hydroxycinnamic acids) and polysaccharides (cellulose and hemicellulose). Phenolic substances like lignin and p-hydroxycinnamic acids, which include ferulic and p-coumaric acids, prevent access to polysaccharides in cell walls (Zhang et al., 2019). The chemical substance, notably lignin, is vital, but the anatomical framework, especially as it gets more complicated with plant age, significantly influences the digestibility of a cell wall (Visković et al., 2024).

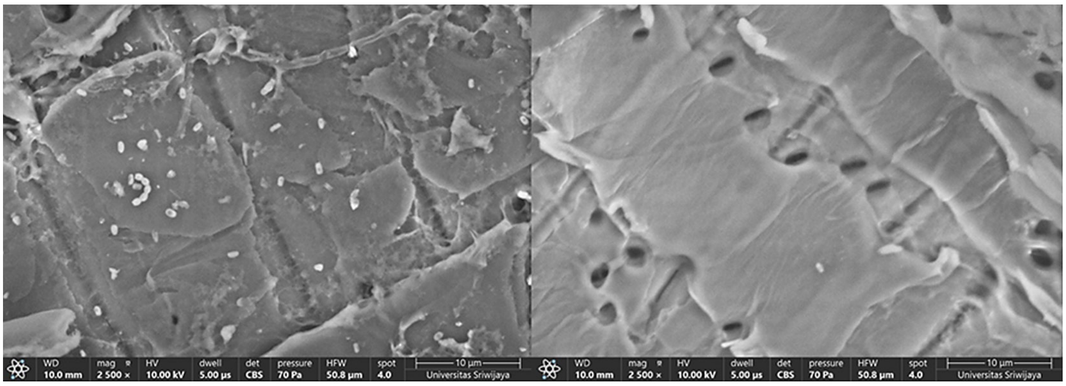
The morphology of untreated and KOH-pretreated rubberwood powder was examined by SEM, as seen in Figure 1. Thermal pretreatment

Table 1. The composition of rubber wood sawdust before and after pretreatment

Components	Untreated rubber wood sawdust % (w/w)	KOH pretreated rubber wood sawdust % (w/w)
Cellulose	35.00	55.26
Hemicellulose	22.00	16.13
Lignin	29.83	13.49

Table 2. EDS examination of the element component of untreated and pretreated rubber wood sawdust (ZAF method, standardless quantitative analysis)

Element	Untreated rubber wood sawdust (% weight)	KOH pretreated rubber wood sawdust (% weight)
C	39.90 ± 0.20	40.90 ± 0.20
O	58.10 ± 0.40	57.50 ± 0.40
Mg	0.40 ± 0.10	0.20 ± 0.01
Al	0.60 ± 0.01	0.30 ± 0.10
K	0.60 ± 0.10	0.00



(a) (b)

Figure 1. Scanning electron microscopy (SEM) images of rubberwood sawdust samples: (a) untreated sample; (b) microwave-assisted alkaline pretreated sample. The magnification of the image provided in Figure 1 is 2500x

induces alterations in the surface's structure. This occurrence promotes the biomass structure's degradation, facilitating microbial access to cellulose (Novia, Aji, et al., 2025). The SEM picture in Figure 1a illustrates a robust adhesion among the original fibers and the non-cellulosic components, including the outer layer of natural rubberwood powder, as shown by Shimizu et al. (2018). The matrix of polymers, as shown in Figure 1b. The breakdown of lignin and hemicellulose components is demonstrated by the processed rubberwood sawdust's cell wall breaking down. The same trend was seen in earlier examinations of injured palm tree trunks (Singh et al., 2022). Prior study (Kundu et al., 2021) showed that the fibers were mostly composed of robustly structured cellulose fibers. The untreated fibers changed their rigid conformation after the matrix components' chemical interactions, which led to the creation of free OH groups. SEM analysis demonstrated the formation of fibrillation and the deterioration of the rubberwood sawdust's stiff matrix.

XRD analysis

XRD analysis is used to determine the material's crystalline and amorphous structures; in this case, it is most likely lignocellulosic biomass (Chorfa et al., 2022). Both unpretreated and pretreated samples could be analyzed since the crystallinity of the cellulose substrates considerably impacted the hydrolysis rate. The XRD shapes of the samples taken both before and after processing are described in Figure 2. For the Untreated sample, the Peak is less sharp and lower, and contains more amorphous fractions (hemicellulose and lignin). Then, the low crystallinity and cellulose are still trapped in the lignin/hemicellulose matrix. In the pretreated sample, the peak is higher and sharper at $2\theta \approx 22.5°$, indicating an increase in cellulose crystallinity. Then the peak at 16° also increases, strengthening the indication of crystalline cellulose enrichment. Additionally, the amorphous peak becomes weaker, signifying that KOH has removed lignin and hemicellulose, and microwave treatment (Dong et al., 2019). After being treated with KOH, the intensity peaks rose while the amorphous components significantly decreased. For both untreated and processed rubber wood sawdust, the intensity peak occurred at 1256 cps. Microwave-assisted KOH pretreatment effectively eliminated amorphous lignin, as seen by the results, which revealed a higher crystallinity index for pretreated samples.

FTIR spectroscopy analysis

FTIR spectra from both untreated and treated samples were used to analyze the composition and presence of functional compounds in rubber wood sawdust, as shown in Figure 3. The first peak between 3400 and 3500 cm^{-1} showed the OH stretch band linked to alcoholic compounds in rubber wood sawdust (Siddiqi et al., 2022). The breaking of the hydrogen chains in cellulose is shown by the reduced intensity at the OH oscillation (Hemansi et al., 2020). The deepest level of the unpretreated sample was inferior to that of the pretreated sample, indicating that lignin breakdown and hydrocarbon production transpired throughout denaturation. The stretching vibrations of the $-CH_2$ and $-CH_3$ groups were confirmed by the 2042 and 2154 cm^{-1} peaks. The peak forms in the 1289–1699 cm^{-1} spectra revealed the reduced

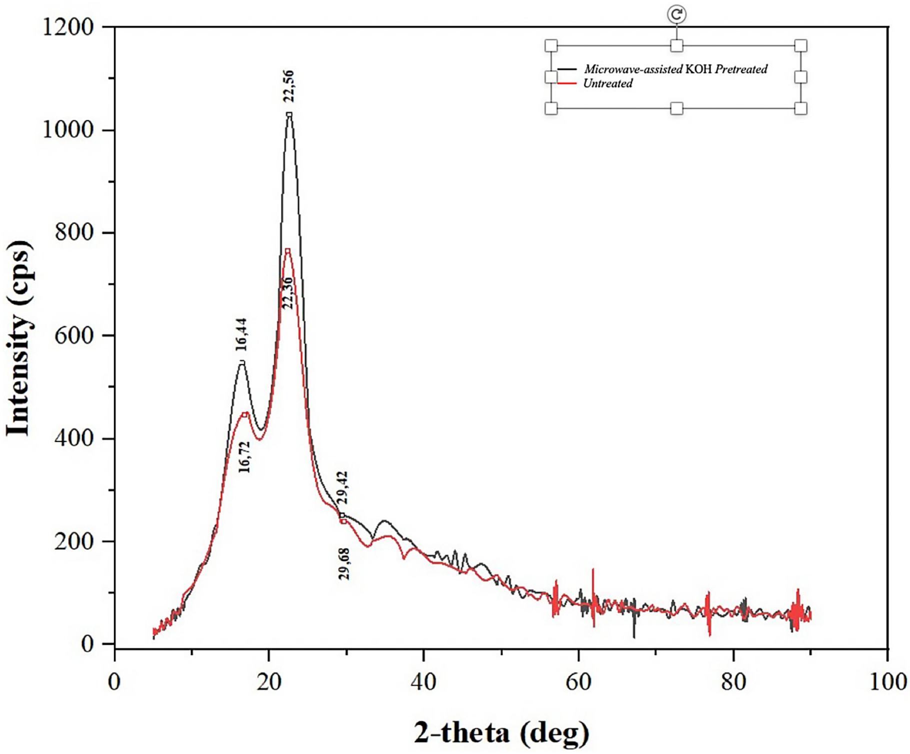


Figure 2. The XRD patterns for unpretreated and pretreated rubber wood sawdust

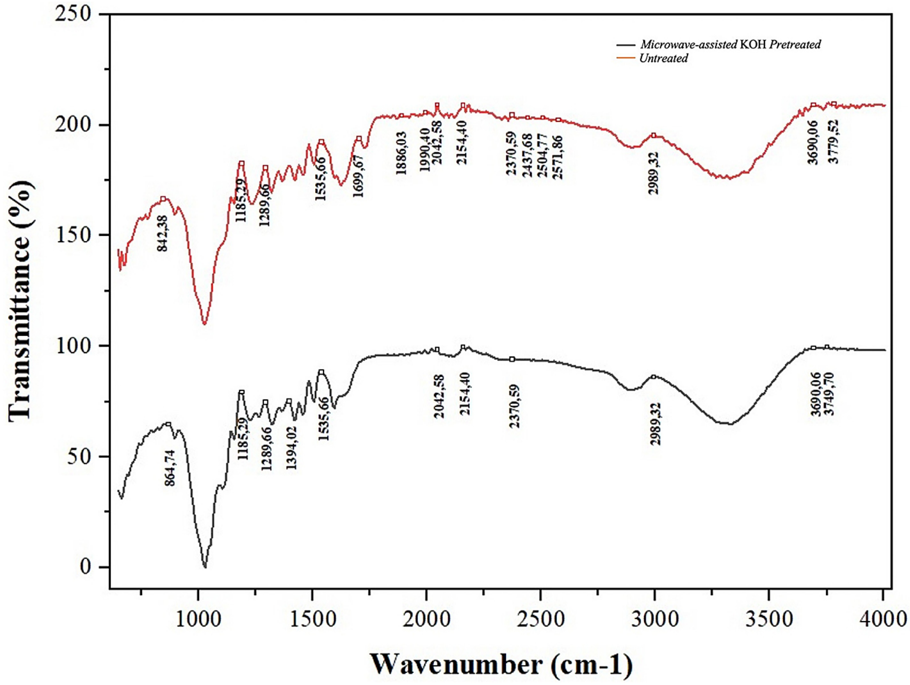


Figure 3. FT-IR analysis of unpretreated and pretreated samples

C=O group from the aromatic compounds in rubber wood sawdust. Between 1185 and 1289 cm^{-1}, a medium stretch zone was found, which is suggestive of lignin alkene compounds (Menon et al., 2018). The cellulose content of rubber wood sawdust revealed a notable decline in minerals over a number of peaks in the 842–1160 cm^{-1} range, which was ascribed to C-O bond vibrations. The peaks in the 2980–3486 cm^{-1} range showed that the hydrocarbons and cellulose in the pretreated samples had dissolved. This demonstrated that there were considerable changes in the molecular structure of rubber wood sawdust between the processed and untreated samples. The results of SEM and elemental analysis were confirmed by FTIR, which indicated that rubber wood sawdust processed with microwave-assisted KOH pretreatment showed an extra significant breakdown. Here, it is evident that lignin and hemicellulose were successfully and extensively eliminated by pretreatment. The KOH pretreatment in a microwave works well to lower the amount of lignin and hemicellulose. Enzymes can more easily access cellulose as a result, improving the efficiency of the next fermentation or hydrolysis process (Liang et al., 2021).

Cellulase enzyme characterization

A 96-hour fermentation process using a strain of Aspergillus niger produced the cellulase enzyme. Together, the three different types of cellulase enzyme complex: endo-β-glucanase, exo-β-glucanase, and β-glucosidase hydrolyze cellulose to produce reducing sugars, mostly glucose. The amount of enzyme needed to convert one micromole of cellulose into reducing sugars per minute was measured in U mL^{-1}.

The CMCase approach and total enzyme activity evaluation were the two main methods used to measure cellulase activity. The activity of endo-β-glucanase in the arbitrary cleavage of cellulose molecules was determined using the CMC case approach. Exo-β-glucanase was added to the broken-down findings to produce cellobiose, which was further hydrolyzed to produce glucose. Concurrently, the activity of exo-β-glucanase in glucose synthesis was measured using the Whatman No. 1 filter paper substrate.

The amount of reducing sugars produced throughout the procedure was determined by analyzing cellulase activity with the Dinitrosalicylic Acid (DNS) procedure. Reducing sugars and DNS combined to produce an orange color that a spectrophotometer could detect. The activity of the cellulase enzyme and the amount of soluble protein were evaluated in this investigation. The total amount of protein in the enzyme solution was measured using the Bradford method.

The study demonstrated the ability of the cellulase enzyme to hydrolyze cellulose into reducing sugars. A spectrophotometer was used to quantify the data colorimetrically. FPase and CMCase activity reached 255.1672 U mL^{-1} and 235.9105 U mL^{-1}, respectively.

Different approaches of measuring cellulase activity might yield different results. Many factors affect the activity of crude cellulase enzymes made from farmed residue, but in particular, the amount of cellulose, the kind of substrate, the medium, the amount of substrate, pH, and temperature (Efrinalia et al., 2022).

Effect of hydrolysis time and enzyme-substrate ratio on the reducing sugar concentration

Figure 4 illustrates the test findings regarding the impact of hydrolysis duration on lowering amounts of sugar at different enzyme doses. According to Figure 4, the overall amount of sugar decreased significantly from 1 to 10 hours. The rise diminishes from 10 to 25 hours, with the peak decreasing sugar levels at 3.15 g L^{-1}. The activity of the cellulase enzyme has entered a moderate phase and is depleting, resulting in a reduction in reducing sugar levels. During an intermediate phase, cellulose undergoes substantial hydrolysis to yield reducing sugars. The quantity of enzymes increases in direct proportion to the level of reducing sugars as enzyme activity intensifies.

After determining the kinetics of enzymatic hydrolysis substrates using a modified Michaelis equation, the models were validated using data from the laboratory. Because the extra substrate limited hydrolysis, the enzymatic hydrolysis was carried out in an unsteady-state environment. It is crucial to consider the shift in the enzyme-substrate complex's concentration under such circumstances. The chemical constituents of the reactant, the product, the temperature, the catalyst, the concentration of substrate, and other variables all affect the reaction's kinetics. Equation 18 was used to calculate the reaction's kinetics. The connection between $[P]/\ln\{[P]\infty/([P]\infty - [P]\}$ and $[E]0.t/[P]/\mathrm{In}\{[P]\infty/[P]\infty - [P]\}$ at different enzyme doses is depicted in Figures 5–10.

Rubberwood sawdust undertook a delignification procedure as a pretreatment process to remove its lignin content, creating cellulose that is more accessible to enzymes throughout hydrolysis. The composition data for sawdust before and throughout pretreatment are revealed in Table 3.

The enzymatic hydrolysis kinetic model determines the amount of reducing sugar released from rubber sawdust pretreated through a hydrolysis time. Linear regression yields reaction kinetics, with the slope denoting the reaction rate constant

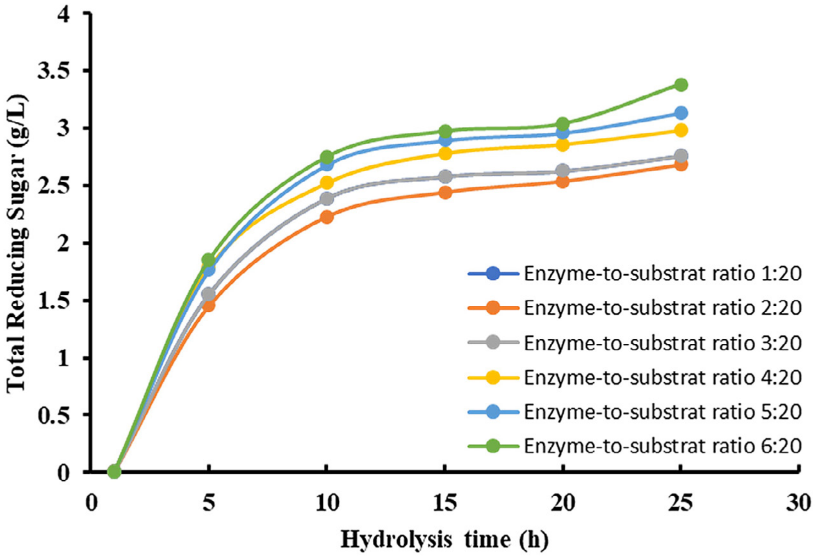


Figure 4. Variations in the total reducing sugar level in the hydrolysate throughout the enzymatic hydrolysis of pre-treated sawdust at different enzyme doses. All tests were conducted in duplicate

Table 3. The kinetic constant value of each enzymatic hydrolysis

Enzyme-to-substrate ratio	K_M (Mol L^{-1})	V_M (Mol L^{-1} s^{-1})	k_2 (s^{-1})	R^2
1:20	0.0052	6.50×10^{-07}	0.7891	0.6094
2:20	0.0052	9.42×10^{-07}	0.5719	0.6159
3:20	0.0052	1.29×10^{-06}	0.5224	0.6306
4;20	0.0052	1.67×10^{-06}	0.5059	0.6454
5:20	0.0051	2.15×10^{-06}	0.5227	0.6580
6:20	0.0055	2.67×10^{-06}	0.5393	0.6498

Table 4. Types of delignification, sugars, and kinetic parameters of enzymatic hydrolysis for various feedstocks

Materials	Pretreatment types	Sugar Product	Kinetic parameter of enzymatic hydrolysis			Ref.
Pistia stratiotes through	Alkaline pre-treatment	Reducing sugar = 31.06 g/L	-	-	-	(Mann et al., 2024)
Moso bamboo	Microwave assisted alkaline deep eutectic solvents treatment inducing	Reducing sugar = 51.97 g /100 g	-	-	-	(Liu et al., 2025)
Brewer's spent grains	Ultrasound-assisted pretreatment	Reducing sugar = 33.2 ± 3.1 g/100 g	-	-	-	(Silva et al., 2025)
Parthenium hysterophorus	Ultrasound-assisted alkaline pretreatment	Reducing sugar = 325.51 mg/gmg/g	-	-	-	(Kumar et al., 2023)
Turfgrass pruning	Thermo-Carbide slag pretreatment	Reducing sugar = 269.55 mg/g	k = 0.026 - 0.097	Fractal dimension (h) = 0.604 - 0.683	-	(Tao et al., 2018)
Corn cobs	Hydrogen peroxide-microwave assisted	Reducing sugar = 15.46 g/L	k_2 = 0.0530 (s^{-1})	K_m = 0.0127-0.0253 (Mol L^{-1})	$Vmax = 1.08 \times 10^{-7} - 3.77 \times 10^{-7}$ (Mol L^{-1} s^{-1})	(Novia, Syaputra, et al., 2025)
Banana stem	KOH Microwave-Assisted Pretreatment	Reducing sugar = 17.69 g/L	k_2 = 0.5482 (s^{-1})	K_m = 0.0037-0.0079 (Mol L^{-1})	$Vmax = 1.28 \times 10^{-6} - 2.76 \times 10^{-6}$ (Mol L^{-1} s^{-1})	(Novia et al., 2024)
Rubberwood sawdust	microwave-assisted alkaline pretreatment	Reducing sugar = 3.15 g/L	k_2 = 0.5393 (s^{-1})	$K_{m =}$ 0.0051-0.0055 (Mol L^{-1})	$Vmax = 6.50 \times 10^{-7} - 2.6 \times 10^{-6}$ (Mol L^{-1} s^{-1})	This Research

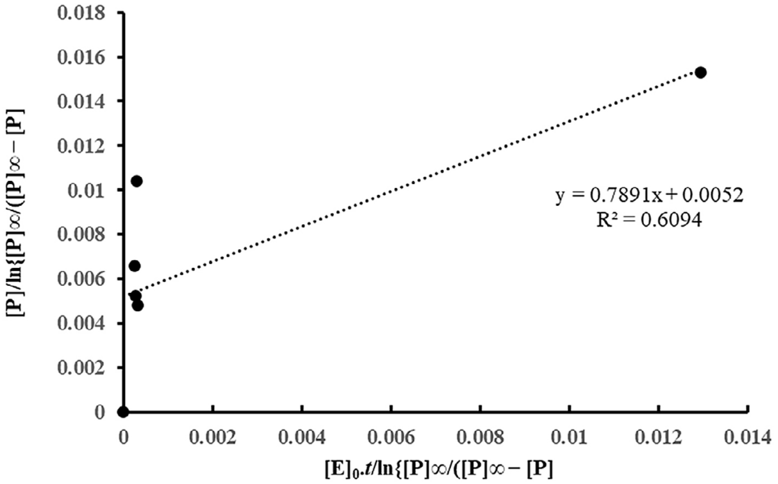


Figure 5. The correlation between [P]/ln{[P]∞/([P]∞ − [P]} and [E]0.t/ln{[P]∞/([P]∞ − [P]} at an enzyme to substrate ratio of 1:20

k_2 and the intercept indicating the Michaelis-Menten constant (K_M). The reaction rate constant k_2 is around 0.5059–0.7891 s^{-1}. The V_M value indicates the degree of saturation of the enzyme reactant. Conversely, Km designates the catalyst's efficacy or a measure of the enzyme dissociation constant. The Michaelis-Menten constant is 0.0051–0.0055 mol L^{-1}, and V_M is 6.5×10^{-7} to 2.67×10^{-6} mol L^{-1} s^{-1}. It can be concluded that K_M is relatively constant in the range of 0.0052, indicating that the enzyme affinity for the substrate does not change much up to enzyme-to-substrate ratio of 5:20, then decreases slightly at enzyme-to-substrate ratio of 6:20. V_M increases with increasing enzyme volume, indicating that the more enzymes, the faster the reaction occurs. R^2 is best at an enzyme-to-substrate ratio of 5:20 (0.658), indicating the data fits the model best. k_2 is highest at an enzyme-to-substrate ratio of 1:20. However, with increasing enzyme volume, it then increases slightly again after at an enzyme-to-substrate ratio of 5:20, indicating that the efficiency per enzyme molecule decreases as the amount of enzyme is increased (possibly due to substrate saturation or the influence of viscosity/other physical effects). Based on Table 3, the enzyme-to-substrate ratio of 5:20 is the most optimal for overall efficiency and reaction rate. If the main goal is efficiency per enzyme molecule, then a ratio of 1:20 is better. However, a ratio of 5:20 is the best choice for a higher productivity and model stability balance. These values indicate that cellulase catalyzes rubber sawdust cellulose into sugar. The reaction rate constant, Michaelis-Menten constant, and product creation rate in this work are comparable to those created in earlier studies (Efrinalia et al., 2022; Novia, Aji, et al., 2025) (Table 4).

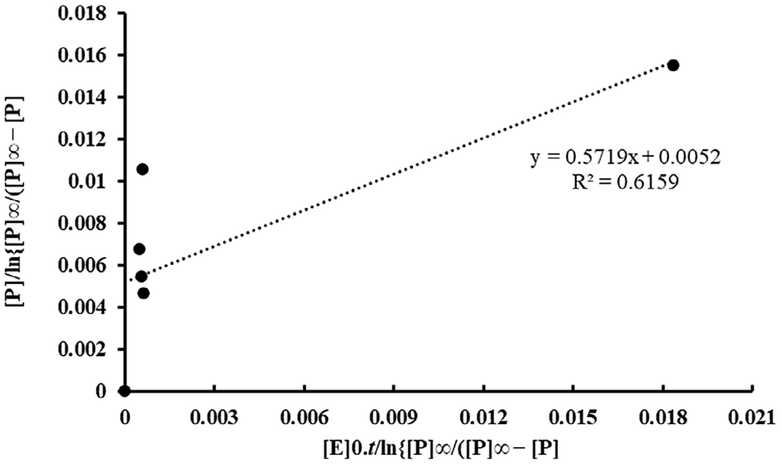


Figure 6. The correlation between [P]/ln{[P]∞/([P]∞ − [P]} and [E]0.t/ln{[P]∞/([P]∞ − [P]} at an enzyme to substrate ratio of 2:20

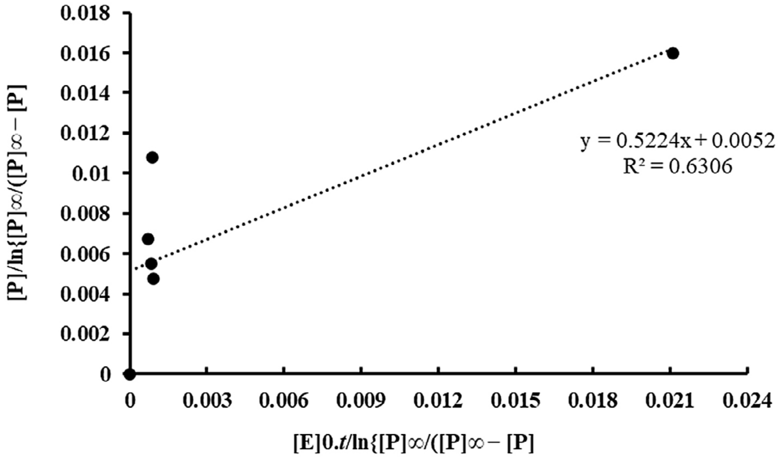


Figure 7. The correlation between [P]/ln{[P]∞/([P]∞ – [P]} and [E]0.t/ln{[P]∞/([P]∞ – [P]} at an enzyme to substrate ratio of 3:20

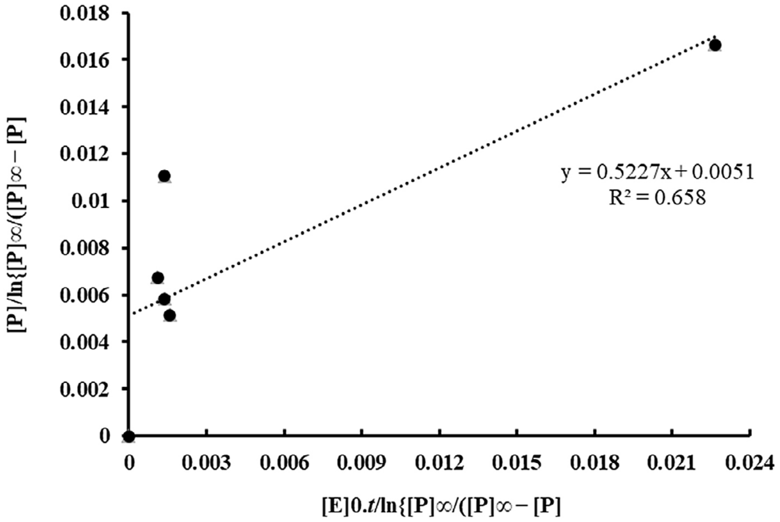


Figure 8. The correlation between [P]/ln{[P]∞/([P]∞ – [P]} and [E]0.t/ln{[P]∞/([P]∞ – [P]} at an enzyme to substrate ratio of 4:20

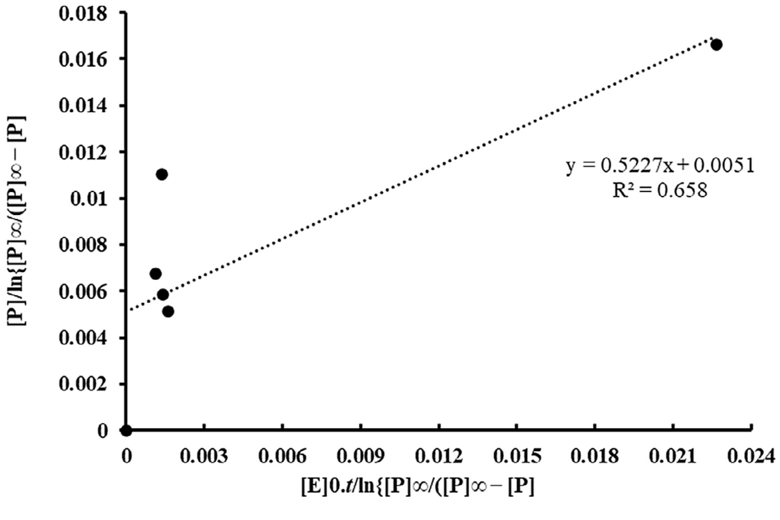


Figure 9. The correlation between [P]/ln{[P]∞/([P]∞ – [P]} and [E]0.t/ln{[P]∞/([P]∞ – [P]} at an enzyme to substrate ratio of 5:20

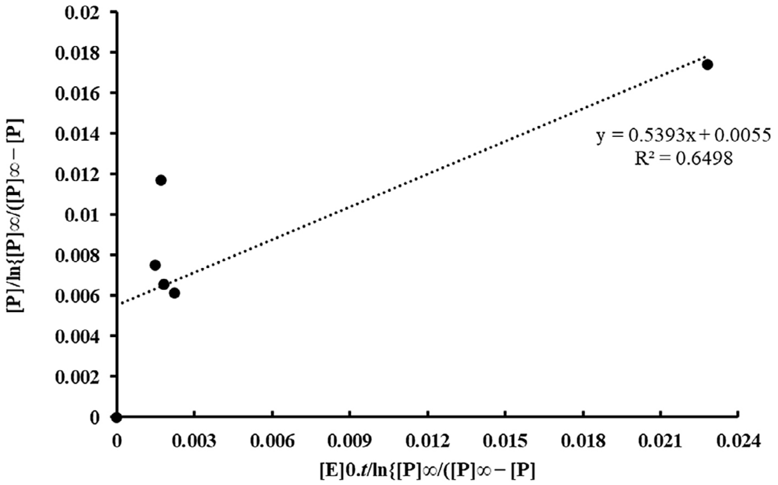


Figure 10. The correlation between [P]/ln{[P]∞/([P]∞ – [P]} and [E]0.t/ln{[P]∞/([P]∞ – [P]} at an enzyme to rubber wood ratio of 6:20

CONCLUSIONS

The endoglucanase activity of Aspergillus niger's cellulase enzyme is 235.9105 U mL^{-1}, whereas the exoglucanase activity is 255.1672 U mL^{-1}. Following pretreatment, the kinetics of the cellulosic breakdown of cellulose from rubberwood sawdust were investigated. The kinetic equation conceivably explains cellulase activity. By changing the enzyme-substrate ratio, the temporal change of reducing sugars was investigated. The research's conclusions led to the development of a novel method for the enzymatic hydrolysis of sawdust from prepared rubber wood that followed the Michaelis-Menten kinetic model. The determined kinetic variables, K_M and V_M, were 6.50×10^{-7} to 2.6×10^{-6} Mol L^{-1} s^{-1} and 0.0051 to 0.0057 Mol L^{-1}, respectively. The results indicated a clear correlation between the concentration of reducing sugar and enzyme loading. At a hydrolysis time of 25 hours and an enzyme-substrate ratio of 6:20, the maximum quantity of reducing sugars (3.15 g L^{-1}) was obtained. More investigation is essential to create an advanced model of cellulose enzymatic hydrolysis, which will be a valuable resource for future industrial applications.

Consequently, informed by the comprehensive pretreatment methodology of rubberwood sawdust (5% KOH, 25 minutes in microwave) and the discrepancies in hydrolysis rates across different enzyme-to-substrate ratios, forthcoming research endeavors to augment biomass hydrolysis efficiency will predominantly concentrate on the genetic engineering of the pretreatment process (encompassing variations in KOH concentrations, microwave durations) or the modulation of hydrolysis rates under diverse pretreatment conditions and fermentation for bioethanol production. In the future, biorefineries must combine insights from pretreatment and enzymatic hydrolysis into conversion processes to create bioethanol from biomass cheaply and efficiently, therefore improving total biomass processing efficiency.

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