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Research Article

Acid–Base Pretreatment and Enzymatic Hydrolysis of Palm Oil Mill Effluent in a Single Reactor System for Production of Fermentable Sugars

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Palm oil mill effluent (POME) is one of the main agro-industrial wastewaters in Malaysia. Highly polluting POME is a serious threat to the environment. In recent years, the methods used to treat POME are inefficient and complex in terms of cost or environmental preservation. The main object of this research is to propose a single reactor system (SRS) obtained from POME wastewater discharge as a promising low-cost treatment and high-energy method for harvesting the fermentable sugar by applying acid–base–enzyme pretreatment and hydrolysis of POME by locally produced cellulase enzymes to enhance biofuel production. Several experiments were conducted to produce fermentable sugars through the statistical methods, including the characterization of POME, acid-base pretreatment, and enzymatic hydrolysis process for reducing sugar production. The one-factor-at-a-time (OFAT) results showed that the highest reducing sugar yield, 23.5 mg/mL of POME, was achieved by enzymatic hydrolysis in an SRS without having a separation and purification. Based on OFAT performance, optimization of two factors such as substrate concentration (total suspended solids, TSS %w/v) and enzyme loading (μmol/min) was carried out by applying face-centered central composite design (FCCCD) under the response surface methodology (RSM) to develop a second-order regression model. The optimum reducing sugar production was 26.6 mg/mL (53.14%) with the conditions of 5% w/v, TSS, and 80 μmol/min/mL of the enzyme dose. In addition, the results of this research can be further considered in biofuel production using other wastewaters to enhance biofuel production as well as wastewater treating functions and minimize the negative environmental impacts.

1. Introduction

Palm oil mill effluent (POME) is being produced in huge quantities during the extraction and purification processes by Malaysia's palm oil mill industry in 2022. More than 429 palm oil mills in Malaysia produced about 64.99–112.91 million tons of POME from 2009 to 2022 [1]. The newly generated POME is a colloidal suspension that contains 95–96% water, 4–5% total solids, and 0.6–0.7% oil, with a high chemical oxygen demand of 53,630 mg/L, oil and grease content of 8,370 mg/L, and biochemical oxygen demand (BOD) of 25,000 mg/L [2]. According to estimates, 5–7.5 tons of water are needed to produce one ton of crude palm oil, and more than half of that water will be converted into POME, which is the main cause of environmental pollution in Malaysia [3].

Currently, the most common treatment method for POME is the ponding process, with more than 85% of palm

oil mills in Malaysia implementing it. However, other techniques such as physicochemical treatment, aerobic and anaerobic digestion, and membrane filtering could also provide useful ideas for enhancing POME treatment procedures in the palm oil industry [2, 3]. In contrast, the anaerobic digestion of POME offers the quickest return on capital due to the ability to collect biogas to be utilized in the production of heat while also using the treated effluent for landfill disposal [2]. Finally, it is proposed that to achieve sustainable development, wastewater mediating effects on the development of green and sustainable biotechnologies and cleaner production should be given the highest priority and integrated into Malaysia's POME monitoring. POME can be reused for biotechnological means since POME contains a higher concentration of organic contents such as protein (12.75%), carbohydrates (29.55%), nitrogenous compounds (26.39%), and minerals [4]. Nevertheless, the traditional system needs long retention times and huge treatment zones because this system typically consists of acidification, de-oiling tank, facultative ponds, and anaerobic with particular hydraulic retention times of 1, 4, 45, and 16 days. Also, the treated POME utilizing the existing system sometimes could not meet the discharge standard of 50 mg/L BOD [5].

Lignocellulosic biomass is a sustainable resource that attends as the cornerstone for the growth of the biorefinery. Biomass is a renewable feedstock with unique properties that make it a content source for creating bioproducts, fuels, and energy [5]. These characteristics include consistent supply, widespread availability, ease of accessibility, and overall production. Meanwhile, the idea of biofuel production is not new, and there have constantly been examples of traditional biorefineries. Instances of biorefinery use at a large scale include industrial growth connected to livestock farming and the pulp and paper sector [6]. Notwithstanding, a more comprehensive and sophisticated understanding of this idea has recently emerged, and today's biorefineries are an important tool in the growth of the bioeconomy [7]. They are designed to provide a wide variety of product investments from a broad spectrum of biomass resources to address the various needs of consumers. However, compared to fossil fuels, bioenergy is much less carbon-intensive. For example, compared to traditional diesel, the oxidation of biodiesel reduces emissions of unburned hydrocarbons, carbon monoxide, and smoke by 20%, 30%, and 50%, correspondingly [3]. It is necessary to emphasize from the standpoint of climate change that since the carbon released during the burning of biofuels is of bioactive composition, it does not participate in the lengthy carbon dioxide, usually defined as the aquifer's greenhouse effect [8]. For example, the utilization of inorganic diesel results in the release of 2.67 kg of CO_2 from an aquifer's origin, which may be completely avoided by utilizing biodiesel [4].

To identify workable ways for controlling POME, sporadic research has been done. The management of POME has already been discussed, with a focus mostly on the current treatment approaches used by palm oil companies. Different pretreatment methods are continuously being developed to enhance the financial and technological use of lignocellulose in biorefineries, for example, in the issue of cellulosic biofuel production [9]. Pretreatment methods have diverse modes of operation and overall effects when applied to various lignocellulosic biomass. A rise in cellulose ease of access and surface area, a reduction in cellulose content and lignin concentration, and neither of these changes would result in a material loss of fermentable sugars [10]. Furthermore, not all pretreatment techniques equally succeed in these objectives. In contrast, the lack of a uniform pretreatment technique forces us to develop novel strategies that combine several pretreatment methods.

To increase the yield of fermentable sugars in the specific situation of cellulosic ethanol, it is imperative to further reduce pretreatment costs. The utilization of lignin as a raw material for industrial applications and the exploitation of pentoses from hemicelluloses (the "lignin platform") are further key factors for the complete industrialization of biofuel production methods [5]. Therefore, having a high lignocellulosic content and carbohydrates, producing sugars, biofuel from POME is one of the best ways to treat it. To ferment POME into biofuel, pretreatment and hydrolysis of the lignocellulosic material have to be done. These processes are commonly done using concentrated and dilute acid, base, and hydrolytic enzymes. Concentrated acid gives high yields but requires large amounts of acid [11]. This poses problems when it comes to neutralization and sugar recovery in addition to its being harmful to the environment. Enzymatic hydrolysis, on the other hand, is simply too expensive yet to be commercialized. Current research development and relevant kinds of the literature showed that various pretreatment methods (acid/base) and hydrolysis processes are applied separately with different separation processes such as filtration, washing, centrifugation, etc., which deals with high processing costs at scale-up production in the industry [12]. To overcome these problems, a single reactor system (SRS) where all pretreatments and hydrolysis processes could be taken in a single pot experiment for enhanced fermentable sugar production towards biofuel fermentation.

In this study, chemical pretreatment (acid and base) was applied in the bioconversion process as they are most efficient in degrading the hemicellulose and lignin layer to make the cellulose more accessible. Therefore, an SRS where acid–base pretreatment and enzymatic hydrolysis were being used when most of the composition in the POME was degraded into fermentable sugars [13] in a single pot without any separation processes is to be considered. To make the economic feasibility of large-scale production, cellulase enzyme was produced locally by using *Trichoderma reesei* where palm kernel cake (PKC) was used as a basal medium. The optimization methods (one-factor-at-a-time [OFAT] and face-centered central composite design [FCCCD]) are applied to evaluate the processes of pretreatment and hydrolysis for maximum production of fermentable sugars [13].

2. Materials and Methods

2.1. Materials. POME was collected from Sime Darby Plantation Sdn. Bhd. Carey Island, Malaysia, at the point of discharge to the aerobic ponding system. The sample was

stored in a cold room (4°C) to prevent the growth of fungus on it. The total suspended solids (TSS) of treated samples were observed using the standard methods of the American Public Health Association (APHA) [13], TDS was measured using the Hanna Instruments (HI) method [14], and the chemical oxygen demand (COD) was measured using the HACH method [14]. The reducing sugar and total sugar were determined by the phenol sulfuric acid method [15] with a spectrophotometer at 490 nm, and pH was measured using pH meters. Analytical grade chemicals are used include the following: sodium sulphite, sodium potassium tartrate (Rochelle salt), hydrochloric acid (HCl), carboxymethyl cellulose (CMC), and 3,5 dinitrosalicylic acid (Merck, Germany); acetone (Hmb GLOBAL Chemical, Germany); bacteriological agar, bacteriological peptone, and potato dextrose agar (PDA) (OXOID LTD, England); citric acid monohydrate and phenol (R&M Chemical, UK); COD HR reagent (300–1500 mg/mL) (Hach, USA); ethanol 95% and glucose (anhydrous) (Hmb GLOBAL Chemical, Germany); potassium dichromate (The Science Company, USA); sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) (Fisher Scientific, UK); Tween-80 (Scientific Scientific, UK); and *T. reesei* (Novozyme, Denmark). Data are the average of three replicates.

2.2. Inoculum Preparation. Inoculum preparation for enzyme production was carried out according to the method by Alam et al. [16]. Each of the 7-day-old *T. reesei* fungal culture plates was washed with around 25 mL of sterile distilled water using a bent glass rod for maintaining the consistency of spore concentration. The suspended fungal cultures were then filtered using Whatman No. 1 filter paper to remove the mycelia from the spore suspension. The filtrate was transferred into a 150 mL Erlenmeyer flask and used as inoculum after measuring its concentration (1.5×10^8 to 3×10^8 spores/mL) by hemocytometer.

2.3. Enzyme Preparation. The cellulase enzyme was locally produced in the laboratory at International Islamic University Malaysia (IIUM). For production, PKC (Sime Darby Plantation Sdn. Bhd. Carey Island, Malaysia) was used as a basal medium for *T. reesei* with a media composition of 0.2% T80 as described by Elgharbawy et al. [17].

2.4. OFAT Technique for Hydrolysis. OFAT analysis method was used to evaluate further the most contributing factors affecting the optimum reducing sugar production. This study was conducted to select the best combinations of the factors to obtain optimum reducing sugar production with minimum requirements, by varying one factor and maintaining the remaining factors constant. The factors to be studied were determined based on the most influencing factors such as sulfuric acid, agitation, time, TSS, sodium hydroxide (NaOH), initial enzyme dose, enzyme pH, hydrolysis time, and the final enzyme dose were considered. The different experimental procedures used to carry out the study are described sequentially.

2.5. Acid Pretreatment Process. The POME sample was pretreated with sulfuric acid. The diluted acid was added to a 250 mL Erlenmeyer shake in a flask containing raw POME. At first, 50 mL of raw POME (5.5% TSS) with different concentrations of sulfuric acids such as 0.5, 1, 2, 4, 6, 8, and 12 (% v/v) and different doses, 1, 2, 3, 4, and 5 (% v/v of POME) was observed to increase the production of reducing sugar. The samples were incubated for various intervals of time (30–120 minutes) at room temperature (30 ± 2°C) with shaking at 100–250 rpm. This process was also carried out by the ratio of acid to substrate concentration. The optimum condition was determined by measuring reducing sugars produced from pretreatment. The pretreatment of the POME before hydrolysis was done intensively using an OFAT method, as shown in Table 1.

2.6. Alkaline Pretreatment Process. Base pretreatment was performed in 250 mL Erlenmeyer flasks. The acid-treated POME was again pretreated with sodium hydroxide for further increase of reducing sugar and adjust the pH to 5–7. Whereas the different concentrations of sodium hydroxide solution such as 0.5, 1, 2, 3, 4, and 5 (% w/v) were investigated. The medium was incubated in a shaker at room temperature (30 ± 2°C) with an agitation speed of 150 rpm for 1 hour. Then, the base pretreatment was conducted by adjusting the pH of the POME by adding a different concentration of NaOH ranging from 0.5 to 5% (w/v) and subsequently readjusted the pH ranges from 5 to 7. The pretreatment of the POME before hydrolysis was done intensively using an OFAT method, as shown in Table 2.

2.7. Enzymatic Hydrolysis Process. The enzymatic hydrolysis was performed by using the same 250 mL Erlenmeyer flask where the acid and base pretreatments were done. The activity of the cellulase enzyme was used to evaluate the rate of enzymatic hydrolysis for varying doses of 20–200 μmol/min/mL/50 mL of pretreated POME to evaluate the rate of enzymatic hydrolysis. In this step, the initial cellulase enzyme dose for 60 minutes (20–100 μmol/min/mL^{-1}), enzyme pH (4.5–7), hydrolysis time (6–48 hours), and enzyme dose (40–200 μmol/min/mL) were observed to optimize the process condition. The flasks were shaken at 150 rpm at room temperature (30 ± 2°C). Samples were withdrawn at intervals of 6, 12, 18, 24, 30, 36, 42, and 48 hours, centrifuged at 5000 rpm for 20 minutes, and the supernatant was analyzed for reducing sugars. Table 3 shows the enzymatic hydrolysis of OFAT factors. Besides substrate concentration, cellulase enzyme dose is also to be optimized by FCCCD.

2.8. Statistical Analysis. The process parameters for the pretreatment and enzymatic hydrolysis of POME were carried out in two stages. In the first stage, the factors were verified by the OFAT method to evaluate probable optimum levels. In the second stage, the optimization process done by FCCCD under the response surface methodology (RSM) was employed to describe the nature of the response surface in the experimental design and clarify the optimal conditions of the most significant independent variables.

The experimental design (DOE) and statistical analysis in this study were carried out using the statistical software

Table 1: Acid pretreatment OFAT factors designed.

Factor		Range tested	Fixed parameters
H_2SO_4	Dilution (% v/v)	0.5–12	Agitation 150 rpm, time 60 minutes, room temperature 30°C ± 2, TSS 5.5%.
	Dose (% v/v)	1–5	
Agitation (rpm)		100–250	1% H_2SO_4, 4% H_2SO_4 dose, time 60 minutes, room temperature 30°C ± 2, TSS 5.5%.
Time (minutes)		30–120	1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, room temperature 30°C ± 2, TSS 5.5%.
TSS (%)		1–6	1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, time 60 minutes, room temperature 30°C ± 2.

Table 2: Base pretreatment OFAT factors.

Factor		Range tested	Fixed parameters
NaOH	Dilution (%w/v)	1–5	TSS 5.5%, 1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, time 60 minutes, room temperature 30°C ± 2.
	pH	5–7	

package Design–Expert 10.0 (Stat-Ease Inc., Minneapolis, USA). This software was employed to identify the experimental design for optimizing and validating the experiments. RSM was applied to optimize the hydrolysis parameters to obtain optimum reducing sugar production. The RSM design in the form of a FCCCD was applied to set the DOE. The DOE setup includes two factors, substrate concentration (POME) and enzyme dose. Table 4 shows the variable and their levels for the experimental design of the FCCCD model, which consist of a set of 13 experimental runs with 5 center points (run 1, 3, 5, 10, and 13). Each factor was considered at three levels with the codes of low (−1), medium (0), and high (+1).

The experimental data obtained from the DOE were analyzed using regression analysis to calculate the regression coefficients of the equation. The results were evaluated through analysis of regression coefficient, analysis of variance (ANOVA), F-values, and p-values. The statistical software 10.0 was utilized for all analyses. A second-order quadratic polynomial equation was then fitted to the data by various regression procedures. For a two-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 AB + \beta_4 A^2 + \beta_5 B^2, \quad (1)$$

In the equation, Y represents the predicted response of reducing sugar production (mg/mL); β_0 represents the intercept; β_1 and β_2 represent the linear coefficients; β_3 represents the coefficients of the interaction, and β_4 and β_5 represent the squared coefficients.

3. Results and Discussion

3.1. Characterization of POME. Generally, characterization of POME is essential before starting the optimization because it was highly correlated to the properties of the POME and the optimization process of the sugar production towards bioethanol production in a SRS. From the different reports of characterization, it was observed that the factors might differ between several production units and other seasons. So, the current study of POME characterization was carried out before starting the critical optimization study and was shown in Table 5. It was observed from the results that the raw sample consists of 5.5% (w/v) of TSS, 2.37 mg/mL of total sugar, 2.9 mg/mL of reducing sugar, 44.08% (w/w) of cellulose, 25.5% (w/w) of hemicellulose, and 19.5% (w/w) of lignin, respectively.

Wu et al., [18] defined 11,3191 mg/L of COD, whereas 70,900 mg/L of COD was reported by Wu et al. [19] even though they collected the sample from the same palm oil mill. Rashid et al. [20] reported that 4.73 mg/mL of TSS, 0.153 mg/mL of total sugar, and 2.92 g/L of reducing sugar were found in the POME. However, Norfadilah et al. [21] found 4.7% of TSS, 22 mg/L of total sugar, and 11.26 mg/L of reducing sugar. The content of cellulose, hemicellulose, and lignin was found in the raw POME at 39.56%, 23.33%, and 25.02% [22]. In comparison, another investigation also reported values similar to the present study [23].

3.2. Acid Pretreatment of POME for Reducing Sugar. The effect of sulfuric acid (H_2SO_4) was evaluated with a concentration ranging from 0.5% to 8.0% as a pretreatment reagent to produce reducing sugar shown in Figure 1. It was observed that the highest reducing sugar was found at 8.7 mg/mL when the diluted concentration was 1% (v/v) and the dose was 4% (2 mL in 50 mL POME), whereas fixed time (60 minutes) and agitation (150 rpm) were considered. In this condition, the ratio between acid and water content has a high impact to break down the raw POME for reading sugar production as POME was primarily acidic.

Based on the results shown in Figure 1, the reducing sugar production outlines during pretreatment of POME were obtained using different concentrations of sulfuric acid (H_2SO_4) (0.5%, 1.0%, 2.0%, 4.0%, 6.0%, and 8.0% v/v). Initially, the dilation of 0.5% of the reducing sugar was increased with a dose of 1% (/50 mL). After that, the dilation from 2% to 5% was slightly drops of reducing sugar yield. In this case, low acid formation of lignocellulosic contents was low yield. However, the dilation of 1.0% of the reducing sugar was linearly improved with growing doses from 1% to 4% (/50 mL) then a little bit falls when the dose of 5% (/50 mL). On the other hand, the sugar production was not significantly different for the POME treated with 2%, 4%, 6%, and 8% (v/v) concentrations, whereas the dose was 1% to 5% (/50 mL). Dilute acids can act as catalysts in a restricted hydrolysis process known as pre-hydrolysis. This comprises hydrolyzing the hemicellulosic fraction while leaving the cellulose and lignin fractions largely unchanged. Acids also release protons, which disrupt the heterocyclic ether bonds between the sugar monomers in the polymeric

Table 3: Enzymatic hydrolysis OFAT factors.

Factor	Range tested	Fixed parameters
Enzyme pH	3.5–6	TSS 5%, 1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, time 60 minutes, room temperature 30°C ± 2, 3% NaOH, enzyme dose 80 U.
Hydrolysis time (hours)	6–48	TSS 5%, 1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, room temperature 30°C ± 2, 3% NaOH, enzyme dose 80 U, enzyme pH 5.
Enzyme dose (U)	40–200	TSS 5%, 1% H_2SO_4, 4% H_2SO_4 dose, agitation 150 rpm, room temperature 30°C ± 2, 3% NaOH, enzyme pH 5, hydrolysis time 18 hours.

Table 4: Parameters used in FCCCD with the level of each factor for hydrolysis.

Factor	Units	Low	High
Substrate concentration	%	4	6
Enzyme dose	U	60	100

Table 5: Characterization of the POME.

Parameters	Units	Concentration
Total suspended solids (TSS)	mg/L	55,130
Total dissolved solids (TDS)	mg/L	52,900
Total volatile solids (TVS)	mg/L	48,880
pH	—	4.28
Chemical oxygen demand (COD)	mg/L	50,100
Total sugars	mg/mL	2.37
Reducing sugars	mg/mL	2.9
Cellulose	% w/w	44.08
Hemicellulose	% w/w	25.5
Lignin	% w/w	19.66

chains generated by hemicelluloses and cellulose. When these bonds are broken, various chemicals are released, mostly sugars such as xylose, glucose, and arabinose [24].

The enhancement of POME caused by improved with increasing the concentration of 2%–8% (v/v) to reduce the sugar around 6.7–6.1 mg/mL was slightly dropped because of the low temperature and acidic condition. Other sugars, primarily glucose, are liberated into liquors during the hydrolysis of feedstock. This sugar can also be obtained from the cellulosic fraction or certain hemicellulosic heteropolymers. The glucose content must be determined since this sugar is the primary carbon source for most bacteria. The sulphuric acid concentration affected the glucose level. In the studies with 6% H_2SO_4 at 128°C for 180 minutes, the greatest value was 8.86 g/L [25]. Beyond this concentration, the POME was optimum, which was almost 9 mg/mL of reducing sugar, as observed under saturated at 1% v/v and a dose of 4%/50 mL. On the other hand, the reduced sugar (5.9–6.5 mg/mL) was slightly increased, and after that, it was decreased when applying the 2% (v/v) dilution with different doses of 1%–5% [26]. Initially, different situations were identified when raw POME was treated from 0.5% to 8.0% v/v and a dose of 0.5%/50 mL, where the reducing sugar production of almost 4.5 mg/mL remained constant after rapidly increasing. The results are in agreement with the study described by Kamal et al., [26], where 1% (v/v) was used to produce sugar from POME solid with 8.5 g/L of reducing sugar. Optimum production of reducing sugar from empty fruit bunch (EFB) using acid pretreatment also required 1% (v/v) to yield 12.30 mg/gm [18]. The acid pretreatment is also required to break down the cellulose and hemicelluloses polymers to yield sugars from rice straw, corn cobs, barley straw, and wheat bran that were 49%, 45%, 40%, and 34%, respectively [27]. After acid pretreatment, reducing sugar from POME at a concentration of 11.79 g/L was also detected [28].

3.3. Effect of Time. The breakdown of lignocellulosic material and the byproducts produced through pretreatment periods depended on the different times of incubation at ambient temperature. Figure 2 shows the effect of pretreatment time from 30 to 120 minutes. For this study, the three different diluted concentrations (0.5% v/v, 1% v/v, and 2% v/v) with a dose of 4%/50 mL were applied under the OFAT method to evaluate the performance of the reducing sugar.

As mentioned, these two parameters are fixed from previous parameters such as 1% (v/v) of diluted concentration with 4% v/v of dose and room temperature 30°C ± 2. When the time was recorded at 90 minutes, the highest reducing sugar concentration of 9.0 mg/mL was observed, while keeping the other conditions constant at 1% (v/v) of dilution, 4% (v/v) of dose, 150 rpm of agitation, and room temperature (30°C±2) were considered. However, reducing sugar concentration at 60 minutes was very close to that of 90 minutes, but it was found almost 6 mg/mL at 30 minutes because appropriate retention time was required to remove lignin and hemicellulose by acid pretreatment depending on the different types of lignocellulosic biomass. The results of the current study are in agreement with the described data, which indicated an efficient, reducing sugar production at pretreatment times. According to Kamal et al. [26] and Masami et al. [27], 1-hour pretreatment time was very effective to produce reducing sugar from POME was 11.79 g/L by diluting. Reducing sugar from POME was also optimally provided after 1-hour pretreatment with a maximum reduction of sugar production of 4.0 g/L at 1% (v/v) [5].

3.4. Effect of Agitation. In the OFAT study of agitation, it was established that appropriate agitation should be required to

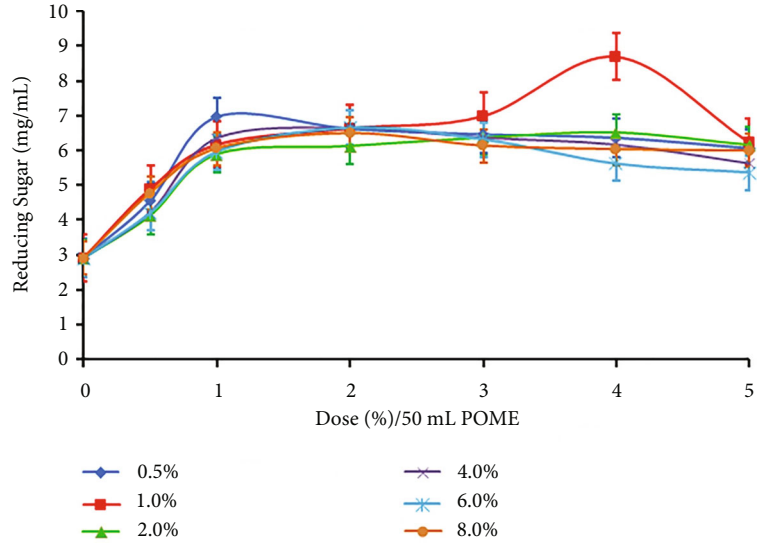

Figure 1: Effect of different H_2SO_4 dilatation and doses on pretreatment. Other factors were fixed at 5.5% TSS, 150 rpm of agitation, 60 minutes of incubation time, and room temperature (30 ± 2°C).

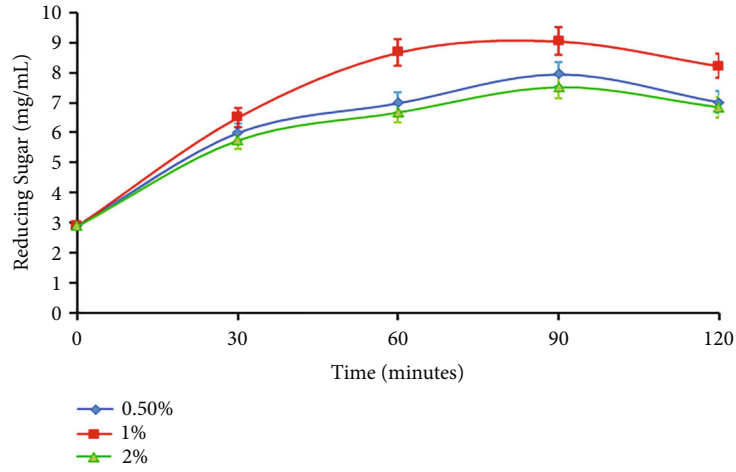

Figure 2: Effect of different pretreatment time on the reducing sugar production. Other factors were fixed at 5.5% TSS, 150 rpm of agitation, 1% (v/v) of diluted concentration H_2SO_4 with 4% v/v of dose, and room temperature 30°C ± 2.

evaluate the pretreatment rate. Figure 3 shows the effect of agitation (50–250 rpm) to develop the reducing sugar production by pretreatment. The result indicated that the maximum reducing sugar was around 9.50 mg/mL after 1-hour pretreatment by 1% (v/v) with 4% v/v dose when 150 rpm was applied, whereas 50 and 250 rpm agitation resulted in the reducing sugar in almost 5.7 and 8.26 mg/mL, respectively, those are lower than 150 rpm.

It was observed from the previous studies that during the pretreatment agitation should be moderate. Many of the researchers described using around 100 rpm of agitation [29] and found 180 rpm of agitation to be optimized for maximum pretreatment of rice straw. In another study, 150 rpm of agitation gave the highest yield of reducing sugar from POME [28] and PKC [18]. The agitation speed is an important parameter for mixing efficiency to raise productivity [30]. Purwanto et al. [29] reported that the lower and higher speed decreased the productivity of the enzymatic hydrolysis process due to the sensitivity of the enzyme to mechanical shear stress. So, the mixing efficiency has been considered in the optimization process. However, the agitation speed was less than 100 rpm, and the amount of sugar yield was lower. On the other hand, at a speed of more than 150 rpm, the amount of sugar yield was maximized [28]. Shah et al. [31] described that moderate agitation was operated to increase the rate of enzymatic hydrolysis, and maximum yield was found at 150 rpm. Several researchers recommended that the optimum agitation for maximum enzymatic hydrolysis of rice straw was approximately 100 rpm [28] found at 180 rpm. Liu and Chen [32] defined that an extremely high agitation speed (>200 rpm) reduced the enzyme activity, whereas a reasonable agitation speed selected from 100–200 rpm is providing a homogeneous mixture for increasing sugar yield and made the hydrolysis rate faster.

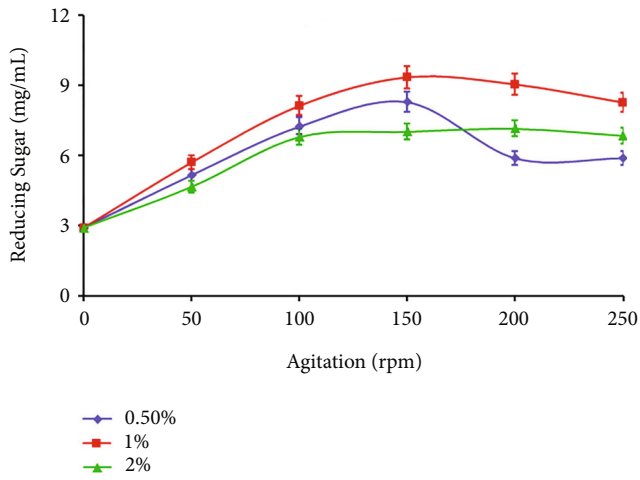

Figure 3: Effect of pretreatment agitation on the reducing sugar production. Other factors were fixed at 5.5% TSS, time 60 minutes, 1% (v/v) of diluted concentration H_2SO_4 with 4% v/v of dose, and room temperature 30°C ± 2.

3.5. Effect of TSS. Several concentrations of TSS (1–6% w/v) were applied to increase the reducing sugar yield by pretreatment, as shown in Figure 4. The maximum reducing sugar yield was observed by TSS of 5% (w/v) with a value of 9.4 mg/mL and after that declined sharply to 6.8 mg/mL at TSS 6% (w/v). As mentioned, other parameters are fixed from previous parameters such as 1% (v/v) of diluted concentration with 4% v/v of dose, 150 rpm of agitation, time 60 minutes, and room temperature 30°C ± 2. The lower TSS (1%) of POME showed very less RS production while it was increased from 2% to 5% TSS. TSS was selected to be an appropriate medium based on the consideration of the dilution factor.

Additionally, a high concentration of TSS might decrease the water content in the reaction mixture, which dropped the yield of reducing sugar [32]. On the other hand, a low concentration of TSS can cause an increase in water content, which also decreased the reducing sugar yield [33]. Based on Siti-Normah et al. [33], reducing sugar was produced at an optimum TSS of 2% (w/v) by using oil palm fronds. Khaw and Ariff [35] reported that the highest production (12.25 g/L) of reducing sugar was obtained at the optimal condition when 150 g/L of POME solid was used. In another study, a higher yield of reducing sugar from EFB was found at 5% (w/v) of TSS [28].

3.6. Alkaline Pretreatment of Acid Pretreated POME. The main function of alkali pretreatment of acid-pretreated POME is to remove the lignin. Lignin is a complicated polymer, and the existence of lignin in pretreatment broth can prohibit the development of microorganisms in the pretreatment process. In this study, alkali pretreatment of acid-pretreated POME was conducted utilizing sodium hydroxide (NaOH) with 5.5% w/v TSS loading. The conditions of pH (pH 5 to pH 7) and concentrations of dose (0.5% w/v to 5.0% w/v) of alkali pretreatment of acid-pretreated POME were shown in Figure 5.

As can be observed in Figure 5, the lignin was break down by increasing the concentration of alkali. Maximum yield of reducing sugar was achieved by 10 mg/mL in pH 6 with 3% w/v of sodium hydroxide dose for the time of 60 minutes, 1% (v/v) of diluted concentration with 4% v/v of dose, 150 rpm of agitation, and room temperature 30°C ± 2. The chemical relationship between cellulose and lignin might has been still quite high under this condition. According to the very low alkali condition of pH 5, the reducing sugar was gradually increased from 0.5% w/v to 3% w/v NaOH dose. The NaOH doses of 3% w/v to 5% w/v resulted in only minor reductions due to the high recovery of the cellulosic fraction in the total suspended solids (TSS). For the high alkaline condition of pH 7, the reducing sugar production was very low compared to conditions pH 5 and pH 6. NaOH was the appropriate solution for higher yield because it required less volume to achieve the optimal pH and it does not affect the medium. The primary process is hemicellulose hydrolysis. In layman's words, acid catalyzes the breakdown of long hemicellulose chains into shorter chain oligomers, which are then degraded by the acid. However, because of the amorphous nature of hemicellulose, less extreme conditions are necessary to liberate hemicellulose sugars. The purpose of the pretreatment procedure is to disturb the crystalline structure of cellulose and break down the lignin structure, so that acids or enzymes may readily access and hydrolyze the cellulose. Pretreatment is necessary for accelerating the single-step hydrolysis process and making the cellulose more accessible. Acid-pretreated lignocellulosic material was affected by alkalis such as NaOH or solution [36], which efficiently developed the enzymatic hydrolysis.

Generally, pretreated lignocellulosic materials were developed with the surface area reachable to cellulase enzyme and redistribution of lignin. Base pretreatment of lignocellulosic materials was more effective to increase surface area, decrease crystallinity, distract the lignin structure, and split-up up structural bonds between carbohydrates and lignin [37]. In general, base pretreatment can be done at room temperature and at different times depending on various lignocellulosic materials. It is defined to cause high sugar degradation than acid pretreatment and it was presented to be less efficient on wood materials than on agricultural residues [38]. From the previous study [34], it was found that pretreatment with NaOH was the most suitable to modify the POME and oil palm fruit fiber (OPFF). The best result was obtained by 3% NaOH pretreatment from EFB and lignocellulosic biomass [28, 33]. Siti-Normah et al., [13] observed that the lignin-to-cellulose ratio for the POME was relatively higher when NaOH was used as a pretreatment reagent. Zhang and Cai [39] also described that alkali pretreated highly influences the enzymatic hydrolysis on rice straw. In the lignocellulosic biomass, alkali pretreatment may provide better performance because it can remove the lignin layer efficiently more than acid.

3.7. Enzyme Hydrolysis of Acid–Based Pretreated POME: Optimization Study. Optimization of the enzymatic hydrolysis was done in two steps. In the first step, three relevant parameters were observed in an OFAT design to evaluate

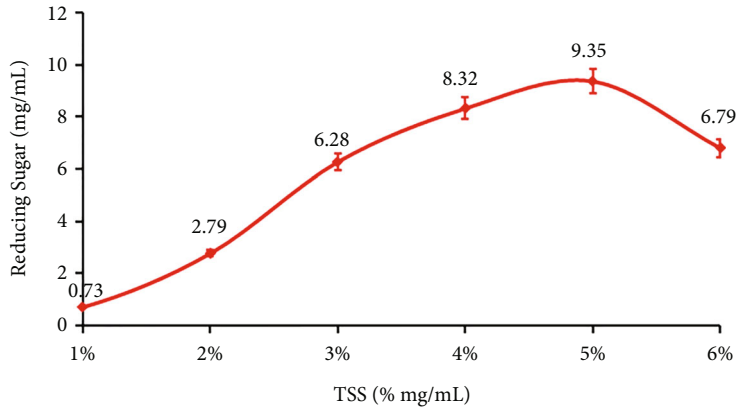

Figure 4: Effect of TSS on the reducing sugar production. Other factors were fixed at time 60 minutes, 1% (v/v) of diluted concentration H_2SO_4 with 4% v/v of dose, 150 rpm of agitation, and room temperature 30°C ± 2.

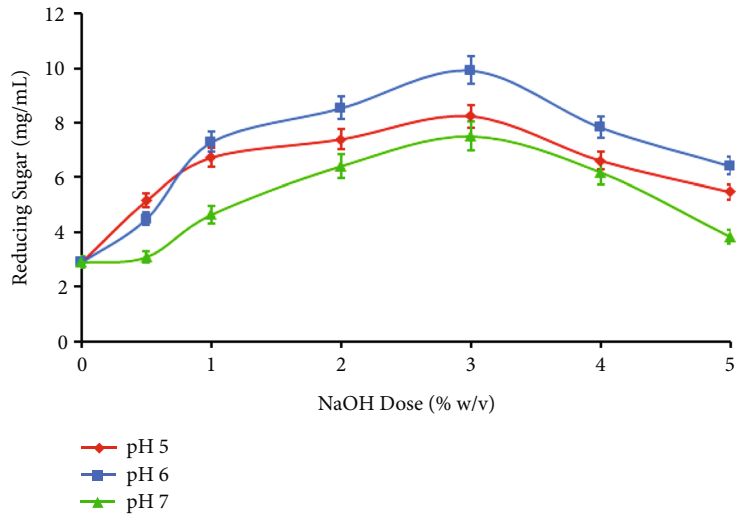

Figure 5: Effect of different NaOH dilatation on pretreatment. Effect of TSS on the reducing sugar production. Other factors were fixed at TSS 5%, time 60 minutes, 1% (v/v) of diluted concentration H_2SO_4 with 4% v/v of dose, 150 rpm of agitation, and room temperature 30°C ± 2.

possible optimum levels of the parameters. The parameters were hydrolysis pH, hydrolysis time, and enzyme dose with the pretreatment strategy of the POME established in the previous sections. In the second step, the parameters found to be optimum from the OFAT study were further observed by the statistical optimization method, FCCCD. The OFAT experimental analysis was further used to investigate the most contributing factors from the pretreatment process for reducing sugar production. This was conducted to obtain optimum design conditions for sugar production before the optimization stage [18]. The important parameters subjected to OFAT studies consisted of enzyme pH, hydrolysis time, and enzyme dose to explore their effects on reducing sugar production. Initial enzyme dose was explained in supplementary materials section, as shown in Figure S1.

3.8. Effect of pH. The effect of the hydrolysis pH on the reducing sugar production of the cellulase enzyme was observed at different pH ranges from pH 4.0 to 6.0 as shown in Figure 6. The optimal pH of this enzyme was established to be pH 5.

At this pH, the reducing sugar production showed 14.5 mg/mL, whereas the control (without enzyme treatment) had 2.9 mg/mL of reducing sugar. Though the optimum reducing sugar production was found at pH 5; however, it was seen that the enzyme reacted by reducing sugar production within the range of pH from 4.5 to 6. The reducing sugar production at pH 4.5 and 6 was below the optimum reducing sugar production at pH 5 by 13.9 and 12.2 mg/mL, respectively. From the literature, it was also observed that the cellulase enzyme from *T. reesei* requiring the optimal pH for the activity of cellulase enzyme was pH 4–6 [41]. Alam et al. [42] reported that the activity of the cellulase enzyme was higher at pH 5. All biochemical and chemical reaction needs a particular hydrogen ion concentration in the reaction environment for proficient execution. It was described that the pH estimation of 5.0 was observed to be perfect for the enzymatic hydrolysis of sunflower

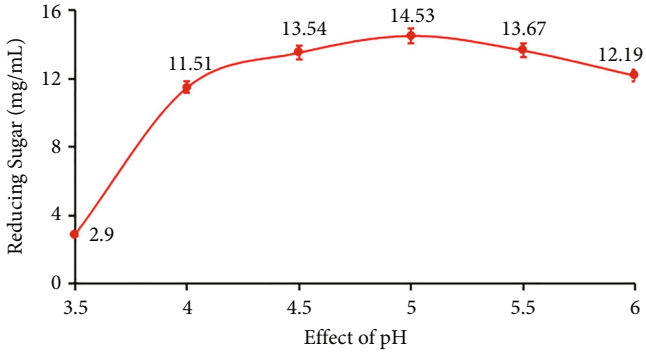

Figure 6: Effect of enzyme pH on hydrolysis. Other factors were fixed at 5% (w/v) TSS, 1% (v/v) diluted H_2SO_4, H_2SO_4 dose 4 v/v %, time 60 minutes, agitation 150 rpm, 3% (w/v) diluted NaOH enzyme dose 80 U, and room temperature 30°C ± 2.

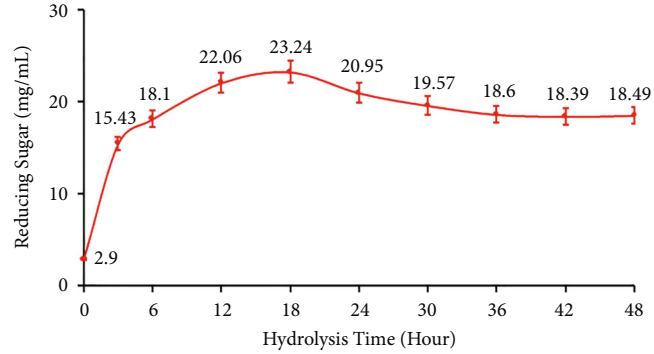

Figure 7: Effect of incubation time on hydrolysis. Other factors were fixed at 5% (w/v) TSS, 1% (v/v) diluted H_2SO_4, H_2SO_4 dose 4 v/v %, agitation 150 rpm, 3% (w/v) diluted NaOH enzyme dose 80 U, and room temperature 30°C ± 2 and pH 5.

stalks. Reduction of hydrolysis was seen at pH values lesser or higher than the optimum [43]. Another specialist Ortega et al. [44] examined the pH impact on hydrolysis in detail and found that CMC was the substrate more affected by acidity. As opposed to insoluble celluloses, CMC has a negative charge that can be adjusted pH of the medium. The difference in the substrate charge can adjust the similarity and catalytic effectiveness of enzymes. The most significant yield of fermentable sugars was found at pH 4.0 after 24 hours of hydrolysis. At this point, when the CMC fermentation was done in 48 hours at acidic pH values, the production of reducing sugars was approximately twice that when utilizing buffer at pH 6.0. Varieties in pH influenced the hydrolysis of insoluble celluloses significantly less, and the most significant yield was found at pH 5 after 48 hours [45].

3.9. Effect of Time. Proteins suffer denaturation and degradation of catalytic activity over time. Besides, some enzymes might be noticeably unstable and lose their activity over a certain incubation time. This optimized process condition was observed through the yield of reducing sugar from 0 to 48 hours of enzymatic hydrolysis. The effect of incubation time on reducing sugar production was studied, in which the amount of reduced sugar produced was determined every 6 hours up to 48 hours. It was found that incubation time influenced reducing sugar production, where maximum reducing sugar was observed after 18 hours of incubation time with 23.2 mg/mL. The results of the examination of hydrolysis time shown in Figure 7 suggested that the reducing sugar yield was not increased by extending the incubation time after 18 hours.

The result of the creation of unnecessary sugar in the medium created an inhibitory effect that limited the hydrolysis rate. The quicker conversion rate of pretreated POME converted to reducing sugar during the initial step of enzymatic hydrolysis may be caused by a smaller element of cellulose favoring the access of the enzyme to possible cleavage sites [43]. Mun et al. [46] found that the reduced sugar production from pretreated POME solid was almost constant after 8 hours. The total reduced sugar content was increased at 96 hours of incubation time by using EFB [17]. On the other hand, Huang et al. [43] studied 100 hours of enzymatic hydrolysis of rice straw, while Ma et al. [47] did the enzymatic hydrolysis of rice hulls for 72 hours. It is essential to know the adjusted incubation time. The activity of each enzyme in a given cellulase preparation is dependent on the enzyme source [17]. The hydrolysis properties of a given cellulase are generally concentrated on kinetic analyses. Kinetic studies are utilized to analyze the cellulolytic limit of enzyme methods from various sources [48], and additionally the contingent enzymatic liability of different cellulosic substrates [49].

Usually, the parallel studies contain an estimate of the time course of hydrolysis over some initial reaction period and also the measurement of the total amount of hydrolysis after a stable, relatively long, reaction period. Through kinetic studies, possible systems of the cellulase are estimated, and a kinetic model can be established, which can be used to predict the rate of hydrolysis of a particular compound [41] under a specific time course to measure the best rate of hydrolysis. It is a practically evident parameter to concentrate on the total hydrolysis of an enzyme system on the balancing substrate. Prajapati and Kango [40] defined 24 hour incubation period to realize the maximum yield of sugar. The yield was reduced before and after 24 hours. Although Aswathy et al. [48] found an increasing trend of hydrolysis to the extent of 72 hours, the rate of hydrolysis declined significantly after 36 hours of incubation. From the above, both researchers observed the waste biomass. On the other hand, Ortega et al. [44] considered the kinetic behavior on three standard cellulosic substrates, which are microgranular cellulose, powdered cellulose, and CMC, and quantified 48 hours to be the best hydrolysis time frame.

3.10. Effect of Enzyme Dose. Different concentrations of the enzyme dose such as 40 μmol/min/mL, 80 μmol/min/mL, 120 μmol/min/mL, 160 μmol/min/mL, and 200 μmol/min/mL are used to optimize the enzymatic hydrolysis. The effect of enzyme dose on hydrolysis was evaluated while keeping other factors fixed at 5% (w/v) total suspended solids (TSS), 1% (v/v) diluted H2SO4, 4% (v/v) H2SO4 dose, 150 rpm agitation, 3% (w/v) diluted NaOH, pH 5, 18-hour

incubation time, and room temperature of 30°C ± 2. It indicates that the reducing sugar has been just above 18.23 mg/mL in the enzyme activity of 40 μmol/min/mL. But it gradually increased up to 23.46 mg/mL of the reducing sugar when using 80 μmol/min/mL of enzyme activity, which was found to be satisfactory. After that, the graph slightly falls pointedly, and the enzyme activity of 200 μmol/min/mL of the reducing sugar was below 20 mg/mL. Generally, enzyme loading was influenced by the activity of the enzyme solution and the resource of enzyme production. The cellulosic materials absorbed the enzyme very quickly after being loaded into the medium. Shah et al. [51] found that during hydrolysis, the loaded enzymes were absorbed by the lignocellulosic materials within 10 minutes. Kim et al. [52] found the optimal condition of enzymatic hydrolysis of food waste having enzyme activity of 400 AG μmol/min/g of *Aspergillus glucoamylase*. Based on the literature review and analyzing the OFAT results methodically, it was selected to study the substrate concentration and enzyme dose by further applying FCCCD.

From the OFAT study, room temperature, 5% (w/v) TSS, 1% (v/v), dose 2 mL, agitation 150 rpm, 3% (w/v) NaOH, pH 5, and hydrolysis time of 18 hours were fixed as an operational condition for the hydrolysis process of POME during RSM study. Since all the enzymes are protein in nature, so they are denatured at a higher temperature. The temperature effect is noticeable as hydrolysis is a process catalyzed by the enzyme alone.

The hydrolysis process was reduced at higher temperatures because of the thermal inactivation of *endoglucanase* and *cellobiohydrolase* [53]. The appropriate temperature was found to be 50°C for enzymatic hydrolysis [54]. Moreover, at temperatures lower or higher than 50°C, less hydrolysis yield was observed. The optimum temperature was also found at 50°C for enzymatic hydrolysis of different lignocellulosic materials [45]. Whereas the optimum temperature for high glucose yield and low enzyme deactivation was found at 35°C [40], 40°C, and 46.3°C [50].

3.11. Statistical Optimization of the Hydrolysis Process by FCCCD under RSM. Central composite design (CCD) and Plackett–Burman Design (PBD) are the two effective and efficient methods for systematic analysis of the target factors. The interface of the parameters, those that were narrowed down through PBD, is optimized considering the quadratic, interaction, and linear effects in the treatment under CCD. Several researchers have utilized these optimization methods to evaluate the maximum production of reducing sugar [30]. From the OFAT studies, the significant factors were imperiled to an optimization process in the form of FCCCD under the RSM method to obtain the optimum enzymatic hydrolysis conditions for maximal reducing sugar yield. During the design, the interaction between factors can be studied. The key factor in the optimization process is to improve and assess the statistical approach to gain a better understanding of the relationship between the factors intricate in reducing sugar production and to reduce the number and cost of experiments.

The FCCCD investigated two factors, substrate concentration (4–6% w/v) and enzyme dose (60 μmol/min/mL to 100 μmol/min/mL), while other factors that had less influence on reducing sugar production were fixed at their respective optimal concentrations determined through OFAT experiments, as previously stated. The actual and predicted values of the reducing sugar for each experimental run were taken from the regression equation of 13 runs, as shown in supplementary materials Table S1. Based on the results, the highest reducing sugar production achieved was 26.57 mg/mL at the center point of the design, and the lowest reducing sugar production observed was 18.34 mg/mL in run 2, as shown in supplementary materials Table S1.

Various regression analyses of the experimental data were used to calculate the regression coefficients of the equation, and the fitted equation was employed to predict the reduced sugar production. The quadratic polynomial equation provided stages of reducing sugar production as a function of substrate concentration and enzyme doses, which can be prepared in terms of code factors, as shown in equation (2):

$$Y = +26.13 + 0.36 \times A - 0.26 \times B + 1.47 \times AB - 4.58 \times A^2 - 2.63 \times B^2, \quad (2)$$

where Y represents the amount of reducing sugar (mg/ml) produced as a function of the coded levels of substrate concentration (A) and enzyme dose (B), respectively.

ANOVA of the response surface, the quadratic polynomial model, is shown in Table 6. The F-value of 258.22 and p-value of <0.0001 of the model indicate that the selected quadratic model was significant. P-value was also employed to explain the significance of each coefficient and utilized to observe the interaction strength between each coefficient that is independent. The lower the p-value, the coefficient becomes more significant. A P-value of <0.01 implies that model terms are significant, whereas values greater than 0.1 mean insignificant model terms. In this case, the terms A, B, and AB were found to be significant factors that had a remarkable influence on the overall reducing sugar production. Meanwhile, based on the F-values of the main factors studied, the substrate concentration presented the highest value, denoting that it shows the strongest influence on reducing sugar production, whereas the enzyme dose showed the least pronounced effect. The lack of fit F -value of 2.01 also implies that the lack of fit is not significant relative to the pure error. A nonsignificant lack of fit indicated that the model fits adequately.

The coefficient of determination (R^2) is near one that ensures a better correlation between the actual and predicted values. Furthermore, the efficiency of the model was displayed by the high value of (0.9946) and adjusted to (0.9904). The signal-to-noise ratio was evaluated by adequate precision, in which a ratio greater than 4 is considered a good model, and the model studied demonstrated a ratio of 43.203. In the meantime, the coefficient of variation (C.V.) defines the degree to which the data were distributed. The C.V. for reducing sugar production was 1.39%, which was within the acceptable range of small values of C.V. (close to zero) giving better reproducibility. A high C.V. implies a large variation in the mean value and does not generate a

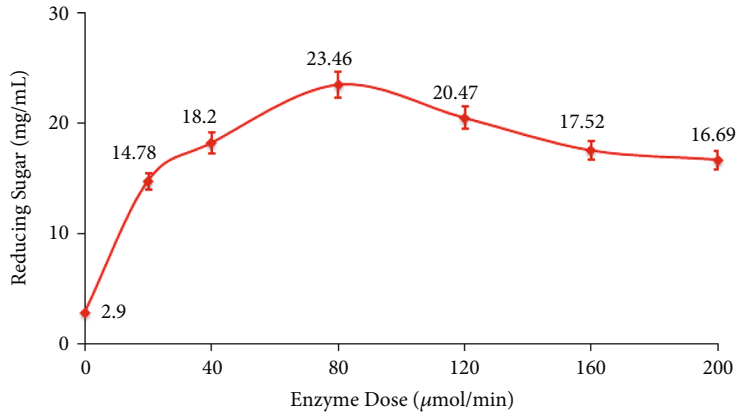

Figure 8: Effect of enzyme dose on hydrolysis. Other factors were fixed at 5% (w/v) TSS, 1% (v/v) diluted H_2SO_4, H_2SO_4 dose 4% v/v, agitation 150 rpm, 3% (w/v) diluted NaOH, pH 5, time 18 hours, and room temperature 30°C ± 2.

Table 6: Analysis of variance (ANOVA) of the polynomial model.

Source	Sum of squares	DF	Mean square	F-value	p-value > F	
Model	129.82	5	25.96	258.22	<0.0001	Significant
A-POME	0.77	1	0.77	7.66	0.0278	
B-enzyme	0.41	1	0.41	4.09	0.0830	
AB	8.64	1	8.64	85.96	<0.0001	
A2	58.03	1	58.03	577.07	<0.0001	
B2	19.16	1	19.16	190.51	<0.0001	
Residual	0.70	7	0.10			
Lack of fit	0.42	3	0.14	2.01	0.2553	Not significant
Pure error	0.28	4	0.070			
Cor Total	130.53	12				

$R^2 = 0.9946$, adjusted $R^2 = 0.9908$, C.V. = 1.39, predicted $R^2 = 0.9651$, adequate precision = 43.37.

satisfactory response model [20]. The regression equation was employed to construct the contour (two-dimensional) and response surface (three-dimensional) plots utilized to examine the interaction between substrate concentration and enzyme dose to determine the optimum concentration of each factor for maximum reducing sugar production. The plots show that reducing sugar production was increased by the increment of substrate concentration and enzyme dose. The degree of the interactions between the variables is represented by the shape of the contour plots [33]. The three-dimensional (3D) response surface and two-dimensional (2D) contour plot of the interaction between substrate concentration (POME) and enzyme dose are presented in Figure 9.

It was revealed that the reduced sugar yield was increased with the decrease in enzyme dose, but there is a suppression of the yield even though the enzyme dose is increased might be due to the saturation effect [56]. The maximum yield of reducing sugar, 26.57 mg/mL of POME, was obtained with the substrate concentration and an enzyme dose of 5% (w/v) and 80 μmol/min/mL, respectively.

3.12. Validation of the Model Developed: Hydrolysis of POME. The statistical model was performed to verify the optimal results to validate the developed model. The different combinations of predicted values of the parameters were calculated from the developed model. The process condition and combination for the hydrolysis of POME composed of factors of independent variables are shown in Table 7. The predicted and experimental process condition for the hydrolysis of POME was found to be within the percentage error < 10%. So, it can be concluded that the developed model is capable of predicting the yield of hydrolysis of the POME. From this study of the developmental enzymatic hydrolysis process, it was evident that the maximum yield, 45.25%, was achieved due to the reduction of the lignin and hemicellulose layer of the POME during the pretreatment and the interaction of the other important parameters, such as % TSS.

Enzymatic hydrolysis of different lignocellulosic biomasses, like food waste [57], sunflower stalks [55], water hyacinth [38], rice straw [56], etc., were studied. Among them, a 30.3% yield was recorded [55] during saccharifying of rice straw, 57.8% hydrolysis was yielded from sunflower stalks [55], and 32% yield was achieved from rice hulls [56].

4. Conclusions

A novel SRS-based pretreatment and hydrolysis techniques were conducted to develop the reducing sugar production

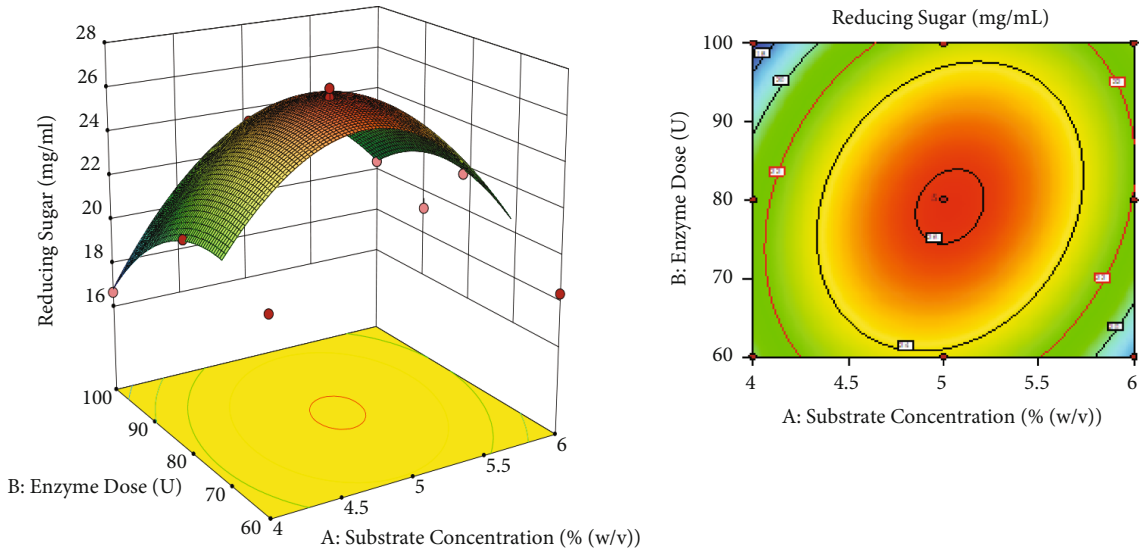

Figure 9: Interaction of POME and enzyme dose on hydrolysis (mg/mL of reducing sugar of POME): (a) 3D response surface; (b) 2D contour plots.

Table 7: Validation of the developed model enzymatic hydrolysis of POME.

Experiment Number	POME (%)	Enzyme (U)	Reducing sugar (mg/mL)		
			Predicted value	Experiment value	Error (%)
1	4	70	21.4	20.94	2.2
2	5.5	90	24.74	22.94	3.34
3	4.5	75	24.9	25.89	−3.82

of POME to break down the cell wall, avoid the complexity of the multi-reactor systems, reduce the cost, and decrease the emissions of greenhouse gasses. POME was chosen due to its valuable composition and its easy availability in Malaysia as an Agra-waste in the palm oil industry. The pretreatment process is more effective on enzymatic hydrolysis for monomeric sugar production. The results exposed that in the study of acid and alkaline pretreatment, the reduced sugar production was improved from 2.9 mg/mL to 12.59 mg/mL. However, the pretreated POME was hydrolyzed by cellulase enzymes in the same reactor to further increase of reducing sugar. The optimization of the process parameters was carried out by applying the OFAT and statistical methods. The highest reducing sugar production was found at 26.57 mg/mL, which is a 53.14% of improvement over the control. In this case, the regression values suggested that the model was significant and fit. The findings show a positive route on efficient management of POME through fermentable sugar production towards biofuel production, which could contribute to the economic development of the country and globally as a whole.

Data Availability

Data supporting this research article are available from the corresponding author or first author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1. Effect of initial enzyme dose on hydrolysis. Other factors were fixed at 5% (w/v) TSS, 1% (v/v) diluted H_2SO_4, H_2SO_4 dose 4%, time 60 minutes, agitation 150 rpm, 3% (w/v) diluted NaOH, pH 6, and room temperature 30°C (± 2). Table S1. Faced-centered central composite design (FCCCD) experimental design for selection of medium components and process conditions for reducing sugar yield. *(Supplementary Materials)*

Effect of Initial Enzyme Dose. The cellulase enzyme activity of 40 CMC μmol/min/mL was initially used for 60 minutes in varying doses from 10–100 μmol/min/mL to evaluate the rate of enzymatic hydrolysis using pretreated POME. Figure S1 shows that the rate of enzymatic hydrolysis and enzyme dose was directly proportional to each other. The highest reducing sugar almost 12.59 mg/mL was found at 80 μmol/min/mL of enzyme dose and lowest reducing sugar about 9.99 mg/mL was found at 10 μmol/min/mL of enzyme dose, respectively.

Faced-Centered Central Composite Design (FCCCD) Experimental Design for Hydrolysis. Expert 10.0 software used to design and validate experiments for optimum reducing sugar production through hydrolysis. Response surface methodology (RSM) employed using face-centered central composite design (FCCCD). Factors with clear optimum levels chosen from maximum reducing sugar production points, while others fixed at highest producing levels. Two factors were analyzed for FCCCD such as substrate concentration and enzyme dose. Table S1 shows the experimental design of the FCCCD model, which consist of a set of 13 experimental runs with 5 center points (run 1, 3, 5, 10, 13). Each factor was considered at three levels with the codes of low (−1), medium (0), and high (+1). Table S1 shows the actual and predicted values of the reducing sugar for each experimental run, taken from the regression equation of 4 runs. Based on the results, the highest reducing sugar production achieved was 26.57 mg/mL at the center point of the design, and the lowest reducing sugar production observed was 18.34 mg/mL in run 2.

References

[1] M. P. O. Board, *Malaysian Palm Oil Statistics*, Economics and Industry Development Division, Kuala Lumpur, Malaysia, 2022.

[2] N. Deb, M. Z. Alam, M. F. Al-khatib, and A. Elgharbawy, "Development of acid–base-enzyme pretreatment and hydrolysis of palm oil mill effluent for bioethanol production," *Liquid Biofuel Production*, pp. 197–217, 2019.

[3] D. Dominic and S. Baidurah, "Recent developments in biological processing technology for palm oil mill effluent treatment—a review," *Biology*, vol. 11, no. 4, p. 525, 2022.

[4] M. H. Silveira, A. R. Morais, A. M. da Costa Lopes et al., "Current pretreatment technologies for the development of cellulosic ethanol and biorefineries," *ChemSusChem*, vol. 8, no. 20, pp. 3366–3390, 2015.

[5] P. Manzanares, "The role of biorefinering research in the development of a modern bioeconomy," *Acta Innovations*, vol. 37, pp. 47–56, 2020.

[6] M. Tabatabaei and M. Aghbashlo, "The critical role of advanced sustainability assessment tools in enhancing the real-world application of biofuels," *Acta Innovations*, vol. 37, no. 37, pp. 67–73, 2020.

[7] A. M. da Costa Lopes, R. M. Lins, R. A. Rebelo, and R. M. Łukasik, "Biorefinery approach for lignocellulosic biomass valorisation with an acidic ionic liquid," *Green Chemistry*, vol. 20, no. 17, pp. 4043–4057, 2018.

[8] H. Kamyab, S. Chelliapan, R. Shahbazian-Yassar et al., "Evaluation of lipid content in microalgae biomass using palm oil mill effluent (Pome)," *Journal of Metals*, vol. 69, no. 8, pp. 1361–1367, 2017.

[9] H. Kamyab, M. F. Md Din, C. T. Lee et al., "Lipid production by microalgae Chlorella pyrenoidosa cultivated in palm oil mill effluent (POME) using hybrid photo bioreactor (HPBR)," *Desalination and Water Treatment*, vol. 55, no. 13, pp. 3737–3749, 2015.

[10] H. Kamyab, M. F. Md Din, M. Ponraj et al., "Isolation and screening of microalgae from agro-industrial wastewater (POME) for biomass and biodiesel sources," *Desalination and Water Treatment*, vol. 57, no. 60, pp. 29118–29125, 2016.

[11] H. Kamyab, M. F. Din, C. L. Tin et al., "Micro-macro algal mixture as a promising agent for treating POME discharge and its potential use as animal feed stock enhancer," *Jurnal Teknologi*, vol. 68, no. 5, 2014.

[12] R. Ochieng, A. Gebremedhin, and S. Sarker, ""Integration of Waste to Bioenergy Conversion Systems: A Critical Review," *Energies*, vol. 15, no. 7, p. 2697, 2022.

[13] APHA, *Standard Methods for the Examination of Water and Wastewater*, pp. 1000–1000, American Public Health Association, and American Water Works Association, Washington, DC, USA, 17th edition, 1995.

[14] Washington DC HACH, *Analysis Handbook*, HACH, Loveland, CO, 4th edition, 2002, 355, 383, 749.

[15] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[16] M. Z. Alam, N. A. Kabbashi, and S. N. I. Hussin, "Production of bioethanol by direct bioconversion of oil-palm industrial effluent in a stirred-tank bioreactor," *Journal of Industrial Microbiology and Biotechnology*, vol. 36, no. 6, pp. 801–808, 2009.

[17] A. A. Elgharbawy, M. Alam, N. A. Kabbashi, M. Moniruzzaman, and P. Jamal, "Evaluation of several ionic liquids for in situ hydrolysis of empty fruit bunches by locally-produced cellulase," *3 Biotech*, vol. 6, no. 2, p. 128, 2016.

[18] T. Y. Wu, A. W. Mohammad, J. M. Jahim, and N. Anuar, "Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes," *Journal of Environmental Management*, vol. 91, no. 7, pp. 1467–1490, 2010.

[19] T. Y. Wu, A. W. Mohammad, J. M. Jahim, and N. Anuar, "Treatment of palm oil mill effluent (POME) using ultrafiltration membrane and sustainable reuse of recovered products as fermentation substrate, in 4th seminar on water management (JSPS-VCC)," *Johor*, pp. 128–135, 2006.

[20] S. S. Rashid, Z. Alam, M. I. A. Karim, and M. H. Salleh, "Development of pretreatment of empty fruit bunches for enhanced enzymatic saccharification," *African Journal of Biotechnology*, vol. 10, no. 81, pp. 18728–18738, 2011.

[21] N. Norfadilah, A. Raheem, R. Harun, and F.'.–. R. Ahmadun, "Bio-hydrogen production from palm oil mill effluent (POME): a preliminary study," *International Journal of Hydrogen Energy*, vol. 41, no. 28, pp. 11960–11964, 2016.

[22] W. K. Mun, N. A. Rahman, S. Abd-Aziz, V. Sabaratnam, and M. A. Hassan, "Enzymatic hydrolysis of palm oil mill effluent solid using mixed cellulases from locally isolated fungi," *Research Journal of Microbiology*, vol. 3, no. 6, pp. 474–481, 2008.

[23] D. P. Singh and R. K. Trivedi, "Acid and alkaline pretreatment of lignocellulosic biomass to produce ethanol as biofuel," *International Journal of ChemTech Research*, vol. 5, no. 2, pp. 727–734, 2013.

[24] Q. A. Nguyen, M. P. Tucker, F. A. Keller, and F. P. Eddy, "Two-stage dilute-acid pretreatment of softwoods," *Twenty-First Symposium on Biotechnology for Fuels and Chemicals*, pp. 561–576, Humana Press, Fort Collins, CO, USA, 2000.

[25] R. Aguilar, J. A. Ramırez, G. Garrote, and M. Vázquez, "Kinetic study of the acid hydrolysis of sugar cane bagasse," *Journal of Food Engineering*, vol. 55, no. 4, pp. 309–318, 2002.

[26] S. A. Kamal, J. M. Jahim, N. Anuar et al., "Pre-treatment effect of palm oil mill effluent (POME) during hydrogen production by a local isolate clostridium butyricum," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 2, no. 4, pp. 325–331, 2012.

[27] G. O. Masami, I. Usui, and N. Urano, "Ethanol production from the water hyacinth Eichhornia crassipes by yeast isolated from various hydrospheres," *African Journal of Microbiology Research*, vol. 2, no. 5, pp. 110–113, 2008.

[28] R. Martínez-Corona, C. C. Penagos, M. del Carmen Chávez-Parga, M. Alvarez-Navarrete, and J. C. González-Hernández, "Analysis of the effect of agitation and aeration on xylitol production by fermentation in bioreactor with Kluyveromyces marxianus using hydrolized tamarind seed as substrate," *International Journal of Current Microbiology and Applied Sciences*, vol. 5, no. 6, pp. 479–499, 2016.

[29] L. A. Purwanto, D. Ibrahim, and H. Sudrajat, "Effect of agitation speed on morphological changes in Aspergillus niger hyphae during production of tannase," *World Journal of Chemical*, vol. 4, no. 1, pp. 34–38, 2009.

[30] S. N. A. Rahim, A. Sulaiman, K. H. K. Hamid, N. A. Edama, and A. S. Baharuddin, "Effect of agitation speed for enzymatic hydrolysis of tapioca slurry using encapsulated enzymes in an enzyme bioreactor," *International Journal of Chemical Engineering and Applications*, vol. 6, no. 1, pp. 38–41, 2015.

[31] S. R. Shah, U. C. Ishmael, J. V. Palliah, M. F. F. Asras, and S. S. B. N. W. Ahmad, "Optimization of the enzymatic saccharification process of empty fruit bunch pretreated with laccase enzyme," *BioResources*, vol. 11, no. 2, pp. 5138–5154, 2016.

[32] Z. H. Liu and H. Z. Chen, "Periodic peristalsis enhancing the high solids enzymatic hydrolysis performance of steam exploded corn stover biomass," *Biomass and Bioenergy*, vol. 93, pp. 13–24, 2016.

[33] M. D. Siti-Normah, S. Sabiha-Hanim, and A. Noraishah, "Effects of pH, temperature, enzyme and substrate concentration on xylooligosaccharides production," *International Journal of Chemical and Molecular Engineering*, vol. 6, no. 12, pp. 1181–1185, 2012.

[34] Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresource Technology*, vol. 83, no. 1, pp. 1–11, 2002.

[35] T. S. Khaw and A. B. Ariff, "Optimization of enzymatic saccharification of palm oil mill effluent solid and oil palm fruit fibre to fermentable sugars," *Journal of Tropical Agriculture and Food Science*, vol. 37, no. 1, pp. 85–94, 2009.

[36] A. M. Shabbirahmed, D. Haldar, P. Dey et al., "Sugarcane bagasse into value-added products: a review," *Environmental Science and Pollution Research*, vol. 29, no. 42, pp. 62785–62806, 2022.

[37] A. M. Rahmani, P. Gahlot, K. Moustakas, A. A. Kazmi, C. S. Ojha, and V. K. Tyagi, "Pretreatment methods to enhance solubilization and anaerobic biodegradability of lignocellulosic biomass (wheat straw): progress and challenges," *Fuel*, vol. 319, p. 123726, 2022.

[38] P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M. J. Negro, "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review," *Bioresource Technology*, vol. 101, no. 13, pp. 4851–4861, 2010.

[39] Q. Zhang and W. Cai, "Enzymatic hydrolysis of alkali-pretreated rice straw by Trichoderma reesei ZM4-F3," *Biomass and Bioenergy*, vol. 32, no. 12, pp. 1130–1135, 2008.

[40] B. P. Prajapati and N. Kango, "Evaluation of alkali black liquor recycling for rice straw delignification and its effect on enzymatic saccharification," *Industrial Crops and Products*, vol. 1, no. 180, p. 114709, 2022.

[41] J. Zhou, Y. H. Wang, J. Chu, Y. P. Zhuang, S. L. Zhang, and P. Yin, "Identification and purification of the main components of cellulases from a mutant strain of Trichoderma viride T 100-14," *Bioresource Technology*, vol. 99, no. 15, pp. 6826–6833, 2008.

[42] N. Saifuddin, A. Saltanat, and H. Refal, "Enhancing the removal of phenolic compounds from palm oil mill effluent by enzymatic pre-treatment and microwave-assisted extraction," *Chemical Science Transactions*, vol. 3, no. 3, pp. 1083–1093, 2014.

[43] C. Huang, C. Liu, X. Sun et al., "Hydrolysis and acidification of waste activated sludge enhanced by zero valent iron-acid pretreatment: effect of pH," *Desalination and Water Treatment*, vol. 57, no. 26, pp. 12099–12107, 2016.

[44] N. Ortega, M. D. Busto, and M. Perez-Mateos, "Kinetics of cellulose saccharification by Trichoderma reesei cellulases," *International Biodeterioration and Biodegradation*, vol. 47, no. 1, pp. 7–14, 2001.

[45] J. Wallace, M. Brienzo, M. P. García-Aparicio, and J. F. Görgens, "Lignin enrichment and enzyme deactivation as the root cause of enzymatic hydrolysis slowdown of steam pretreated sugarcane bagasse," *New Biotechnology*, vol. 33, no. 3, pp. 361–371, 2016.

[46] P. L. Tang, W. L. Hong, C. S. Yue, and S. Harun, "Palm oil mill effluent as the pretreatment solvent of oil palm empty fruit bunch fiber for fermentable sugars production," *Bioresource Technology*, vol. 314, p. 123723, 2020.

[47] S. S. Mahmod, J. Md Jahim, and P. M. Abdul, "Pretreatment conditions of palm oil mill effluent (POME) for thermophilic biohydrogen production by mixed culture," *International Journal of Hydrogen Energy*, vol. 42, no. 45, pp. 27512–27522.

[48] U. S. Aswathy, R. K. Sukumaran, G. L. Devi, K. P. Rajasree, R. R. Singhania, and A. Pandey, "Bio-ethanol from water hyacinth biomass: an evaluation of enzymatic saccharification strategy," *Bioresource Technology*, vol. 101, no. 3, pp. 925–930, 2010.

[49] C. P. Kubicek and E. M. Kubicek, "Enzymatic deconstruction of plant biomass by fungal enzymes," *Current Opinion in Chemical Biology*, vol. 35, pp. 51–57, 2016.

[50] T. Y. Nguyen, C. M. Cai, R. Kumar, and C. E. Wyman, "Co-solvent pretreatment reduces costly enzyme requirements for high sugar and ethanol yields from lignocellulosic biomass," *ChemSusChem*, vol. 8, no. 10, pp. 1716–1725, 2015.

[51] N. Rasit and O. C. Kuan, "Investigation on the influence of bio-catalytic enzyme produced from fruit and vegetable waste on palm oil mill effluent," *In IOP Conference Series: Earth and Environmental Science*, vol. 140, no. 1, p. 12015, 2018, IOP Publishing.

[52] J. K. Kim, B. R. Oh, H. J. Shin, C. Y. Eom, and S. W. Kim, "Statistical optimization of enzymatic saccharification and ethanol fermentation using food waste," *Process Biochemistry*, vol. 43, no. 11, pp. 1308–1312, 2008.

[53] D. W. Rackemann, J. P. Bartley, M. D. Harrison, and W. O. Doherty, "The effect of pretreatment on methanesulfonic acid-catalyzed hydrolysis of bagasse to levulinic acid, formic acid, and furfural," *RSC Advances*, vol. 6, no. 78, pp. 74525–74535, 2016.

[54] H. B. Aditiya, K. P. Sing, M. Hanif, and T. M. I. Mahlia, "Effect of acid pretreatment on enzymatic hydrolysis in bioethanol

production from rice straw," *International Journal of Technology*, vol. 6, no. 1, pp. 3–10, 2015.

[55] S. K. Sharma, K. L. Kalra, and H. S. Grewal, "Enzymatic saccharification of pretreated sunflower stalks," *Biomass and Bioenergy*, vol. 23, no. 3, pp. 237–243, 2002.

[56] H. Ma, W. W. Liu, X. Chen, Y. J. Wu, and Z. L. Yu, "Enhanced enzymatic saccharification of rice straw by microwave pretreatment," *Bioresource Technology*, vol. 100, no. 3, pp. 1279–1284, 2009.

[57] N. H. Zainan, Z. Alam, and M. A. F. Al-Khatib, "Production of sugar by hydrolysis of empty fruit bunches using palm oil mill effluent (POME) based cellulases: optimization study," *African Journal of Biotechnology*, vol. 10, no. 81, pp. 18722–18727, 2011.