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Optimization of Ultrasound-enhanced Subcritical Water Hydrolysis of Oil Palm Empty Fruit Bunch for the Production of Fermentable Sugar

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Abstract

To enhance the hydrolysis to produce fermentable sugar, oil palm empty fruit bunch (OPEFB) has undergone ultrasonication pretreatment prior to subjection to subcritical water hydrolysis. This work aims to optimize the effect of temperature, reaction time, and the concentration of sodium dodecyl sulfate (SDS) as the surfactant, with the primary aim of maximizing sugar production in the subcritical water hydrolysis process applied to oil palm empty fruit bunch (OPEFB). The pretreatment process conditions were optimized using response surface methodology of the central composite design (RSM–CCD). The experimental design includes three factors and levels, with a range of 180–220 °C temperature (X1), 60–80 minutes process time (X2), and 1–5% w/w SDS concentration (X3), an α value of 1.68, and reducing sugar concentration (g/L) as response (Y1). The optimum condition for subcritical water hydrolysis of OPEFB was obtained at 208 °C, 78 minutes, and 2.6% w/w SDS concentration with an expected yield of 6.09 g/L. As a result, reducing sugar produced by enzymatic hydrolysis increased by 324.7% compared to raw OPEFB, with sugar yield of 45.64% after 36 hours. Along these, changes in crystallinity, chemical composition, lignocellulosic functional groups, and morphology were analyzed to determine the impact of the pre-treatment on OPEFB.

Keywords

oil palm empty fruit bunch, response surface methodology, subcritical water, total reducing sugar, ultrasonication

1 Introduction

Increasing energy demands caused by human activity have necessitated the continuous extraction of fossil fuels in order to satisfy the world's energy requirements. The extraction and transformation of fossil fuels into useful materials emanate greenhouse gases and contribute to the critical problem of global warming [1, 2]. In addition, given the finite and limited nature of fossil sources, there is an imperative need to investigate and implement alternative energy sources to ensure a sustainable and dependable energy supply in the future. Utilizing biomass as a replacement for fossil fuels has garnered considerable attention and is regarded as one of the most promising alternatives [3, 4].

According to Palm Oil Analytics, Indonesia is among the largest producers of palm oil worldwide, with the annual production of 34.7 million tons throughout 2019 [5]. Oil palm empty fruit bunch (OPEFB) is a significant byproduct of the palm oil industry, accounting for approximately 21% of the total production [6]. It is composed of 31%–43% cellulose, 23%–35% hemicellulose, and 11%–23% lignin [7]. Cellulose and hemicellulose are two possible monomer sugar sources in OPEFB and can always be utilized to produce valuable goods, such as biohydrogen [8], biogas [9], and bioethanol [10–12].

Cellulose fibers bind to hemicellulose, and lignin coats the cellulose and hemicellulose to form a dense and complex cell wall in biomass. To increase conversion efficiency, it is necessary to anticipate the resistant properties of OPEFB through the application of suitable pretreatment measures. Currently, a variety of pretreatment techniques are being implemented to decompose the dense lignocellulosic structure so that it can be converted into chemical products [6, 13, 14].

Compared to mechanical and microwave pretreatment, ultrasound pretreatment can increase the hydrolysis of cellulose, hemicellulose, and lignin more effectively [15].

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This pretreatment may not convert biomass to sugars, but the increased surface area as well as the change in crystallinity make the pretreated substrate relatively easy to hydrolyze. In the ultrasonic process, the micro-vacuum bubbles produced by vibrations with a frequency greater than 20 kHz continue to increase and eventually break, causing cavitation. In addition, it yielded structural alterations to the biomass and provided enzymes access to the cellulose. To boost hydrolysis efficiency, ultrasound pretreatment is frequently accompanied by various pretreatments, such as chemical pretreatment, organosolv, or eutectic solvent pretreatment [13, 16–18].

On the other hand, subcritical water hydrolysis (SWH) has attracted attention as an eco-friendly solvent and an appealing reaction media for various fields. This method is technically viable for application in acid and enzymatic hydrolysis [19]. It is reasonably inexpensive, environmentally friendly, involves no hazardous solvents along with a rapid reaction rate. Utilizing raw materials such as coconut coir [20, 21], oil palm [22], corn cobs [23], and others, employing SWH for pretreatment in order to acquire reducing sugars has been studied extensively. The degree of hydrolysis and yield according to initial material characteristics (such as cell wall structural and chemical makeup), as well as the composition of the monosaccharides and the type of linkages between them, are some concerns related to the use of agricultural commodities. Because the optimal working conditions may vary depending on the raw material, comprehensive education is required.

This research focuses on optimizing the SWH pretreatment conditions in order to obtain the highest concentration of reducing sugars. Prior research on optimizing sugar production from OPEFB to reduce sugar production with this particular pretreatment combination is limited. Therefore, the Response Surface Methodology of the Central Composite Design (RSM–CCD) will be used to analyze and enhance the effect of time, temperature, and surfactant concentration on sugar production reduction. Afterwards, enzymatic hydrolysis will be conducted under the optimal SWH pretreatment conditions identified.

2 Materials and methods

2.1 Material

This research utilized OPEFB acquired from Pasaman, West Sumatra, Indonesia. The Datta method [24] revealed that the composition of empty fruit bunch OPEFB was 41.7% cellulose, 24.3% hemicelluloses, 10.9% lignin, 22.7% water-soluble extractives, and 0.3% ash. The OPEFB was subsequently cleaned under running water to remove dirt, followed by two to three days of sun drying. The OPEFB was then broken down to a size of 100 mesh (0.154 mm) and dried for 24 hours in an oven at 60 °C.

Chemicals used in this experiment included sodium dodecyl sulfate (SDS) (Sigma Aldrich, China), and dinitro salicylic acid (DNS) reagent which consist of following chemicals: 3.5-dinitrosalicylic acid (Sigma Aldrich, India), sodium hydroxide (Merck, Germany), sodium potassium tartrate (Merck, Germany), and sodium metabisulfite (Sigma Aldrich, Italy). The chemicals used were analytical grade and used without further purification. Cellulase enzyme used in this experiment is from *Trichoderma reesei*, aqueous solution with ≥ 0.7 units/mg (Sigma Aldrich, Germany).

2.2 Ultrasonication pretreatment

6 g OPEFB was combined with 120 mL of 0.05% H_2SO_4 acid (S/L ratio: 1:20) to perform ultrasonic pretreatment. Elma H/C 20 (Germany) ultrasonication equipment with a 35 kHz frequency was employed. The reaction was conducted at 49 °C for 52 min. Afterward, the solution was filtered to separate solids, washed by distilled water, then dried in an oven at 60 °C for 48 h to a constant weight.

2.3 Subcritical water hydrolysis

A total of 5 g OPEFB from the ultrasonication pre-treatment was suspended in 100 mL of distilled water (S/L ratio 1:20). SDS acting as a surfactant, was added in a predetermined quantity and then stirred. The mixture was subsequently transferred into the high-pressure reactor with an initial pressure of 6 MPa. Throughout the reaction, the pressure increased due to the temperature rise induced by the heating element. The reaction proceeded at the temperature and concentration levels specified in the experimental design. The solution was then vacuum-filtered to separate solids from liquids. The pH of the liquid obtained was measured, followed by the determination of total reducing sugar (TRS). The solid obtained was dried in an oven at 60 °C for 48 h until a constant weight was achieved. The overall experimental procedure is summarized in Fig. 1.

2.4 Optimization of subcritical water hydrolysis using RSM

The CCD model, which has three factors and three levels, was used in this study. Three independent variables were observed, including temperature, time, and SDS surfactant concentration, with an α value of 1.68, as shown in Table 1. The α value is measured from the center to the axial (star points).

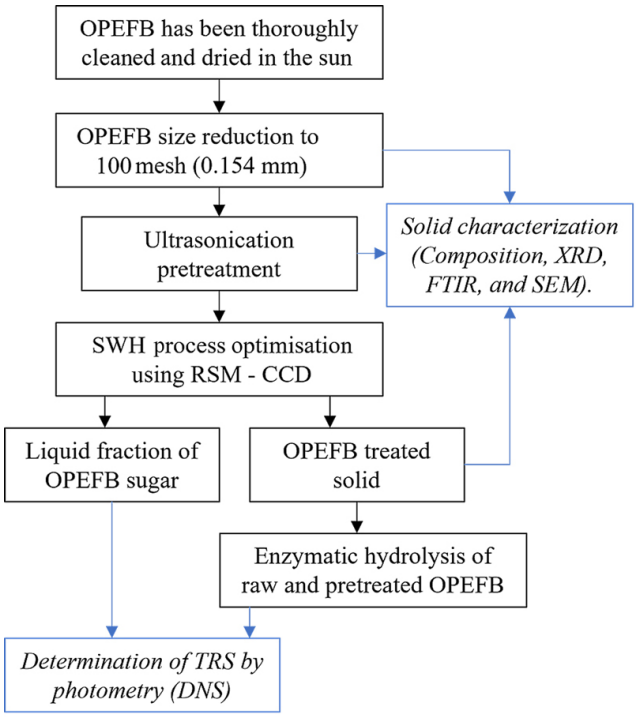

Fig. 1 Flowchart of the experimental method. Italic boxes with blue frame show the characterization of OPEFB includes the solid and liquid from the whole process.

Table 1 Coded and level of process variable CCD with α value of 1.68

Independent variables	Symbol	Coded and actual level				
		$-\alpha$	–1	0	+1	$+\alpha$
Temperature (°C)	X1	166	180	200	220	234
Time (min)	X2	46.4	60	80	100	113.6
SDS conc (%w/w)	X3	–0.4	1	3	5	6.4

The relationship between variables and responses is described by second-order polynomial models, as shown in Eq. 1.

$$y_i = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=2}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad (1)$$

Where y_i represents the response variable, b_0 is the interception coefficient, b_i, b_{ii}, and b_{ij} are the regression coefficients, n is the number of studied variables, and x_i and x_j represent the independent variables.

2.5 Statistical analysis

The statistical significance of individual variable terms was assessed via an analysis of variance (ANOVA) utilizing Minitab 16 (Minitab Inc., ITS Surabaya, Indonesia) [25]. Three-dimensional plots were employed to elucidate the influence of these variables on the response. Subsequently, the model's validity was substantiated through the execution of three supplementary experimental runs under optimized conditions.

2.6 Enzymatic hydrolysis

1 g of OPEFB from SCW hydrolysis was mixed with 30 FPU (1.09 katal) cellulase from Trichoderma reesei, and 30 mL of 0.1 M citrate buffer was added. In a shaker incubator (Fisher 255D, USA), hydrolysis was conducted for 36 h at 50 °C and 125 rpm. Reducing sugar was then measured at the end of the reaction.

2.7 Determination of total reducing sugar

This analysis was conducted employing the DNS reagent in accordance with the method established by Miller [24]. In a 10 mL test tube, 0.2 mL of the hydrolysis solution was added to 1.8 mL of distilled water, which was shaken lightly to homogenize. Next, 3 mL of DNS solution was added and, vortexed for 15 s. The mixture was put into boiling water for 10 min, cooled for 5 min on an ice bath, and allowed to stand until room temperature. The absorbance of the solution was evaluated using a Spectrophotometer (Cecil CE1011, Germany) at 540 nm. The concentration of reducing sugars can then be determined using the standard glucose curve.

2.8 OPEFB solid characterization

OPEFB solids underwent X-Ray diffraction (XRD) analysis to determine changes in crystallinity using X'Pert PRO XRD (PANanalytical BV, The Netherlands); Fourier transform infra-red (FTIR) analysis to determine changes in bonding; and scanning electron microscope (SEM) analysis to determine changes in the morphology of OPEFB using SEM Evo MA 19 analysis (Carl Zeiss, UK).

3 Results and discussion

3.1 Optimization of subcritical water hydrolysis

This study aims to optimize the reducing sugars as a response to subcritical water hydrolysis using the Response Surface Methodology with the Central Composite Design (CCD) matrix design. Compared to traditional methods, the Minitab statistical software optimization process can decrease operating time and costs. This is because the software can assist in achieving optimal results from a series of procedures carried out with high accuracy.

The study consisted of 17 runs with eight cube points, six axial points, and three repetitions at the center point. The research was conducted randomly to minimize errors and data bias. The experiments and results of reducing sugars (Y1) can be seen in Table 2.

Eq. 2 illustrates the quadratic polynomial relationship between the independent variable and the response

Table 2 Design of experiment and response of OPEFB subcritical water hydrolysis

Std Order	Run Order	X1 (°C)	X2 (min)	X3 (%)	[TRS] (g/L)
5	1	180	60	5	3.89
13	2	200	80	–0.4	4.05
9	3	166	80	3	3.47
11	4	200	46.4	3	5.12
17	5	200	80	3	5.76
16	6	200	80	3	5.83
3	7	180	100	1	4.2
2	8	220	60	1	4.81
6	9	220	60	5	4.06
14	10	200	80	6.4	4.14
4	11	220	100	1	5.23
15	12	200	80	3	6.13
1	13	180	60	1	3.02
10	14	234	80	3	5.23
7	15	180	100	5	3.34
12	16	200	113.6	3	4.02
8	17	220	100	5	3.26

Table 3 Analysis of variance (ANOVA)

Source	DF	Adj SS	Adj MS	F-Value	P-Value	Significance
Model	9	13.574	1.507	8.20	0.006	S
Linear	3	3.196	1.065	5.80	0.026	S
X1	1	2.523	2.523	13.73	0.008	S
X2	1	0.189	0.189	1.03	0.344	NS
X3	1	0.483	0.483	2.63	0.149	NS
Square	3	8.229	2.743	14.93	0.002	S
X1×X1	1	4.190	4.190	22.80	0.002	S
X2×X2	1	3.189	3.189	17.35	0.004	S
X3×X3	1	5.512	5.512	29.99	0.001	S
2-Way Interaction	3	2.141	0.713	3.88	0.063	NS
X1×X2	1	0.128	0.128	0.70	0.431	NS
X1×X3	1	0.931	0.931	5.07	0.059	NS
X2×X3	1	1.081	1.081	5.88	0.046	S
Error	7	1.286	0.183			
Lack-of-Fit	5	1.208	0241	6.24	0.144	NS
Pure Error	2	0.077	0.0385			
R-sq: 91.34%			R-sq (adj): 80.2%			

obtained during the subcritical water hydrolysis process after all CCD research works have been completed.

$$\begin{aligned}[TRS] = {} & -81.1 + 0.682X1 + 0.2978X2 + 3.396X3 \\ & -0.001524X1 \times X1 - 0.001330X2 \times X2 \\ & -0.1748X3 \times X3 - 0.000317X1 \times X2 \\ & -0.00853X2 \times X3 - 0.00919X1 \times X3\end{aligned} \quad (2)$$

Eq. 2 illustrates how the correlation between the linear, quadratic, and interaction regression coefficients affects the outcome. Positive coefficients produce outcomes consistent with the response, which means that as the value increases, the response value will increase as well, and vice versa [26]. As can be seen, the response is positively affected by all linear coefficients (X1, X2, X3) but negatively affected by quadratic coefficients (X1×X1, X2×2, X3×X3), as well as interactions (X1×X2, X2×X3, X1×X3).

Table 3 shows the ANOVA result of OPEFB hydrolysis in subcritical water. A factor can be categorized as significant or influential on the response when the p-value is less than 0.05 [18]. As can be seen in the ANOVA table, the model with an F-value of 8.20 is significant with a P-value of 0.006 ($p<0.05$), and a lack of fit is not significant with a P-value of 0.144 ($p>0.05$). This implies that the approach can be used because it can describe the association between variables and the response with minor errors. According to the table, the 2-way interaction model did not produce statistically significant results ($p>0.05$). In contrast, both the linear and quadratic models produced statistically significant outcomes. Nevertheless, the quadratic models, in particular, demonstrated a strong capacity to adequately explain the responses with high significance for each variable ($p<0.05$). This suggests that the nonlinear relationship is more appropriate for describing the association between variables and responses. Additionally, the quadratic model provides insightful information regarding the optimal point or mean value that produces the most favorable response.

Generally, a high fit of the model with the experimental data could be ensured by an R^2 value above 0.8 [27]. In this study, the R^2 value obtained was 91.34 %, indicating that the quadratic model effectively evaluates three variables effect on reducing sugar concentrations. Compared to earlier studies that examined the pretreatment of OPEFB to produce the reducing sugar, this value is higher (R^2 0.72) [5]. This study had smaller errors than previous ones since the evaluation was limited to pretreatment and did not include enzymatic hydrolysis. Consequently, the R^2 value was relatively higher.

The correlation between the residuals and the percent normal probability is depicted in Fig 2. The difference between the actual and estimated response values is known as residuals. Data points close to the normality line indicate that the data are normally distributed, indicating that the results are close to the predictions, confirming an accuracy level of [28].

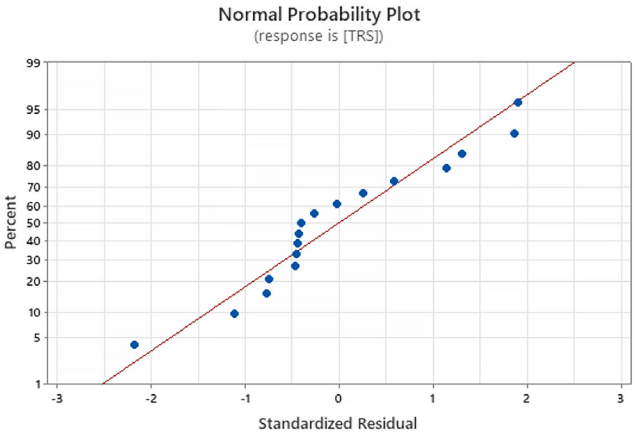

Fig. 2 Normal probability graph

Fig. 3 shows the ideal circumstances suggested by the second-order quadratic model, optimized for the concentration of reducing sugars. The new optimal conditions obtained are 208 °C, 78 min, 2.6% surfactant concentration, and a 6.04 g/L TRS gain. After determining the best combined pre-treatment conditions, the research was carried out to validate the findings. Running validation yielded an average reducing sugar concentration of 5.44 g/L with a relative error of 9.7% from the predicted optimal sugar concentration.

3.2 Effect of pretreatment variables

The impact of the research variables on the response is visually presented in the contour and surface plots shown in Fig. 4 These plots clearly illustrate that as the values of the variables (temperature, time, and SDS concentration) increase, there is a corresponding increase in the yield of reducing sugars, up to a certain limit. However, once that threshold is reached, further increments in the variable values result in a decline in the yield of reducing sugars.

According to the results of ANOVA, the yield of reducing sugars is highly influenced by temperature. Due to the increased diffusivity of the solvent, an increase in temperature improves hydrolysis efficiency, thereby enhancing the solubility of the solute within the solvent. Increasing temperature also modifies the polarity and dielectric constant

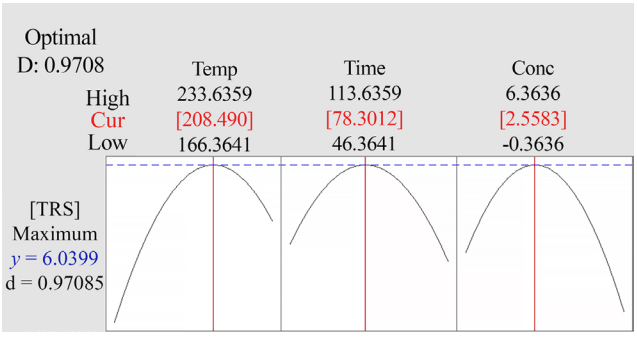

Fig. 3 Optimum condition generated from CCD design

of water, which increases the solubilization of polar compounds such as hemicellulose in water [29]. As the temperature rises, the water becomes more acidic, which weakens the hydrogen bonds between the lignocellulosic fibers. With increase of temperature, the hydrolysis of degraded cellulose and hemicellulose into their derivative compounds rises. As a result, the yield of reducing sugars decreases due to this byproduct.

During the subcritical water period, the matrix cell wall of OPEFB breaks down, which increases the contact area between water and OPEFB, thereby accelerating the mass transfer rate. As with temperature, a longer reaction time promotes the production of byproducts that decrease the reducing sugars yield during subcritical water hydrolysis.

The inclusion of SDS surfactant during the subcritical water process improves the yield of reducing sugars. This enhancement is likely attributed to the formation of surfactant micelles, which consist of hydrophilic and hydrophobic components. The hydrophobic portion plays a role in reducing and eliminating lignin, thereby increasing the accessibility of water to hemicellulose and cellulose. Simultaneously, the hydrophilic portion accelerates the dissolution rate of hemicellulose and cellulose in water. Muharja et al., who studied the recovery of sugar from coconut coir, also found that adding SDS increased the reduction of sugars production [30].

3.2.1 Effect of temperature

Based on the data presented in Table 2, there is a direct correlation between temperature and the amount of reducing sugar produced. For instance, with a reaction time of 60 minutes and a surfactant concentration of 1%, subcritical water pretreatment at 180 °C yielded a reducing sugar concentration of 3.02 g/L. However, when the temperature was raised to 220 °C under the same conditions, the amount of reducing sugar produced increased significantly to 4.81 g/L. When the temperature rises, the ionic constant of water increases, causing water to decompose into H_3O^+ and OH^-, which are responsible for the hydrolysis of OPEFB. Increasing temperature also increases the water availability to cellulose and hemicellulose, resulting in a more excellent production of reducing sugars [31].

3.2.2 Effect of process time

As with temperature, the operating time has a positive impact on the amount of reducing sugar produced because it allows water to hydrolyze lignocellulose into more monomers. However, prolonged reaction times have negative consequences, such as the continued hydrolysis

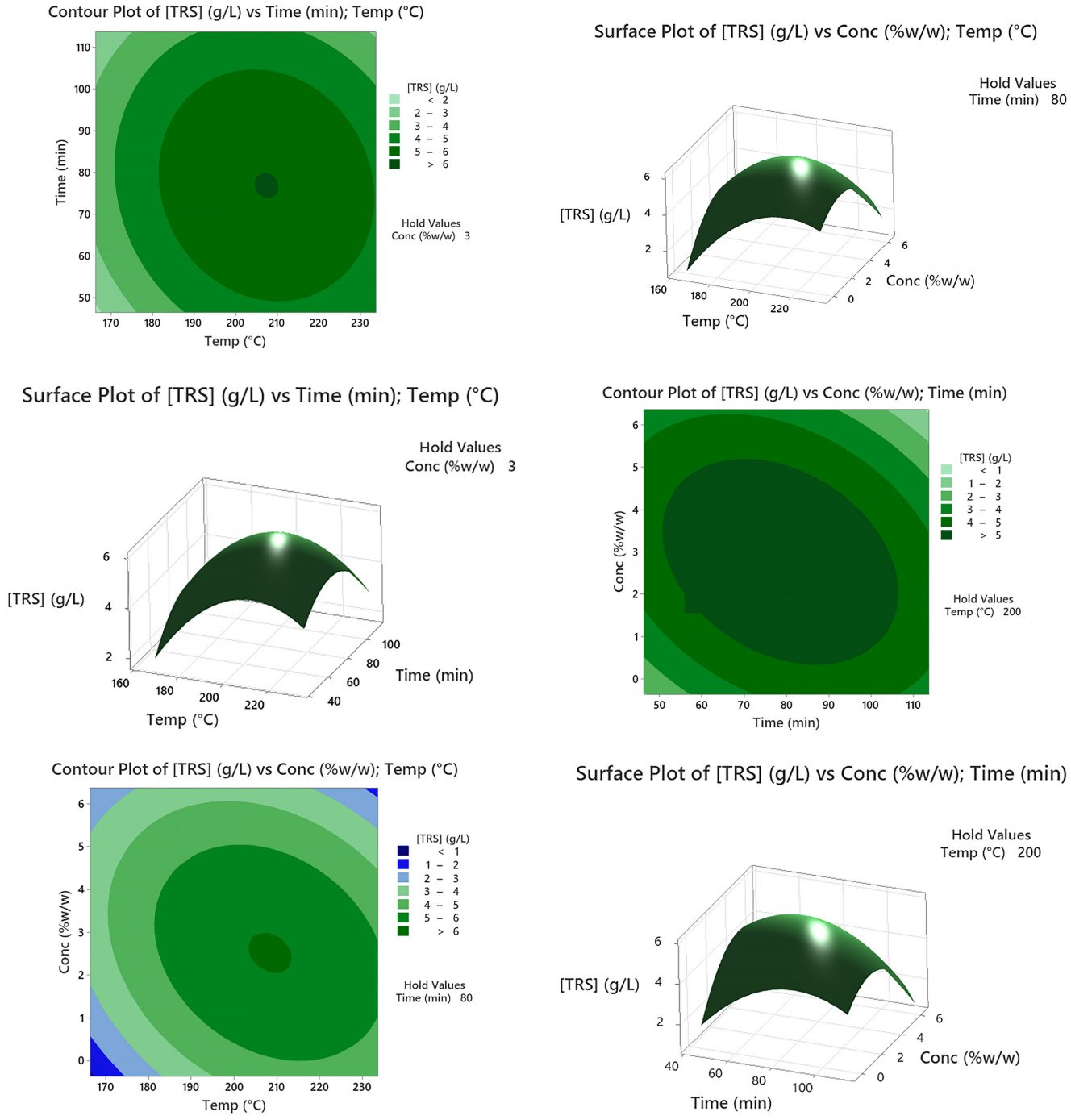

Fig. 4 Contour plot and the surface of experimental variables (temperature, time, and concentration) on TRS

of reducing sugars into inhibitory products. According to Table 2, the reducing sugar yield ranged between 5.76 and 6.13 g/L when the pretreatment was conducted at 200 °C for 80 minutes with a 3% surfactant concentration. This value appears to be higher than the yield observed with a reaction time of 46.4 minutes at the same temperature and concentration, which yielded 5.12 g/L. However, when the reaction time was extended to 113.6 minutes under the same temperature and surfactant concentration, the resulting reducing sugar concentration decreased to 4.04 g/L. This decrease may be attributed to the continued hydrolysis of reducing sugars into inhibitory products during the enzymatic hydrolysis process, such as hydroxymethyl furfural (HMF) and furfural [32].

3.2.3 Effect of surfactant

The use of surfactants aims to enhance the production of reducing sugars. Surfactants form micelles when their concentration exceeds the critical micelle concentration (CMC). These micelles can interact chemically and physically with

both hydrophilic and lipophilic substances [20]. Table 2 demonstrates that, at a processing temperature of 180 °C for 60 min, the addition of 1% surfactant resulted in a reducing sugar concentration of 3.02 g/L, which increased to 3.89 g/L when 5% surfactant was added. However, as the temperature and reaction time were increased, the addition of surfactants led to a reduction in the yield of reducing sugars.

At the midpoint of the experimental design, specifically at 200 °C for 80 min, the addition of 3% surfactant increased the yield of reducing sugars, while the addition of 6% surfactant decreased the yield. This suggests that adding surfactant may not increase the production of reducing sugars under conditions of high surfactant concentration. The effectiveness of surfactant structure in extraction relies on the equilibrium between hydrophobic and hydrophilic forces. Ionic surfactants (such as SDS) form ion pairs with ionic phenolics, which can lead to solution turbidity and a subsequent reduction in sugar production [33]

3.3 Effect of ultrasonication

Fig. 5 demonstrates that ultrasound pretreatment during the initial stages of the subcritical water process is proven to increase the production of reducing sugar. Subcritical water hydrolysis takes place at the previously suggested optimal process conditions (208 °C, 78 min, 2.6% surfactant concentration). Without ultrasound pretreatment, 3.45 g/L of reducing sugar is produced from subcritical water pretreatment. However, with the inclusion of ultrasound pretreatment, the sugar yield notably rises to 5.42 g/L. The increase in reducing sugar following ultrasonic pretreatment was caused by damage to the OPEFB fiber, increasing the fiber's porosity [17]. It makes cellulose and hemicellulose more accessible to water ions. An increase in reducing sugar yield after ultrasonic pretreatment was also reported when using corn biomass as a substrate [34, 35].

3.4 Enzymatic hydrolysis of pretreated OPEFB

Fig. 6 depicts the comparation of reducing sugar produced by enzymatic hydrolysis in OPEFB before and after pretreatment. Enzymatic hydrolysis of OPEFB takes place for 36 h at 50 °C with agitation at 125 rpm. Over the course of 1 to 36 h, the yield of reducing sugars consistently increased. It is evident that the increase in reducing sugar production post-pretreatment is notably higher than without pretreatment. At the conclusion of the reaction, the pretreated OPEFB yielded 9.22 g/L of reducing sugars, marking a 324.6% increase compared to the unpretreated state that yielded 2.84 g/L. This enhancement can be attributed to the deterioration of the morphology of OPEFB fibers, which leads to an expanded fiber surface area due to the removal of hemicellulose and an elevation in its crystallinity index [36]. In subcritical water pretreatment, a portion of the lignin dissolves in water, making cellulose more accessible and facilitating the contact of enzymes with the cellulose surface [37]

Commonly, reducing sugars yield is used to measure the efficiency of enzymatic hydrolysis. The sugar yield from enzymatic hydrolysis is defined as the weight of reducing sugar produced during the hydrolysis process divided by the weight of cellulose in the OPEFB material. Before and after pretreatment, cellulose composition in OPEFB is 43.6% and 60.59%, respectively Table 4. As shown in Fig. 7, the yield value always rises as the hydrolysis period

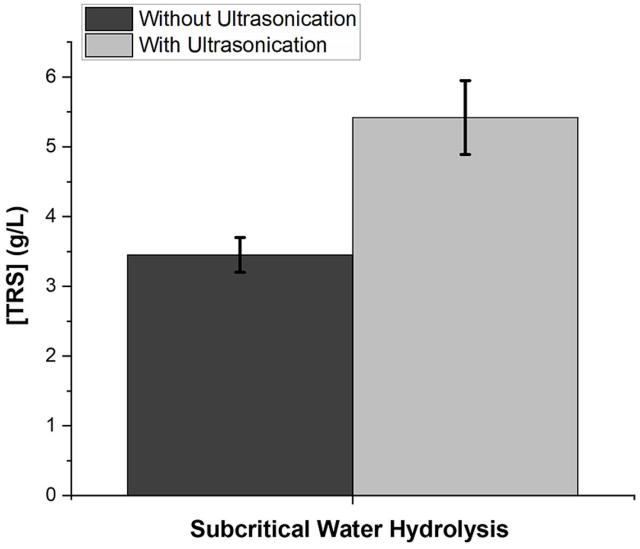

Fig. 5 Comparison of reducing sugars with and without ultrasonication pretreatment on subcritical water hydrolysis

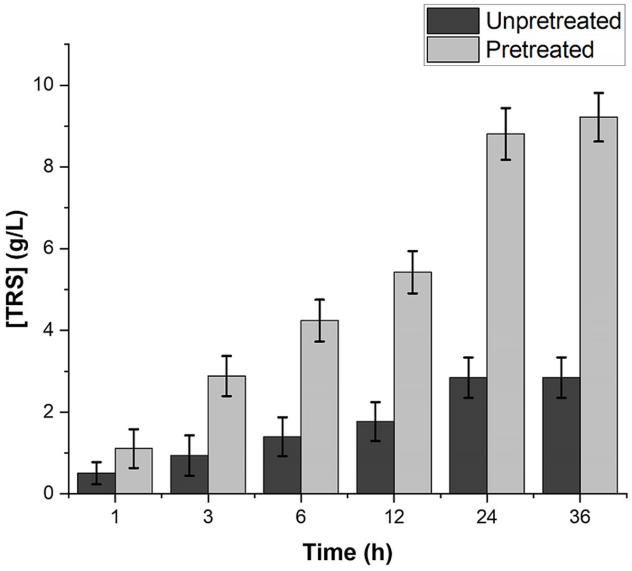

Fig. 6 Sugar produce by OPEFB hydrolysis before and after pretreatment

Table 4 OPEFB composition using the Datta approach [24]

	% Composition*				
	E	H	C	L	A
Unpretreated	18.65	26.78	43.6	10.95	0.31
Ultrasound pretreated	9	27.1	47.88	16.85	0.54
Ultrasound-SWH pretreated	5.82	6.76	67.28	19.23	0.91

*Notes: Extractives (E) ; Hemicellulose (H); Cellulose (C); Lignin (L); Ash (A)

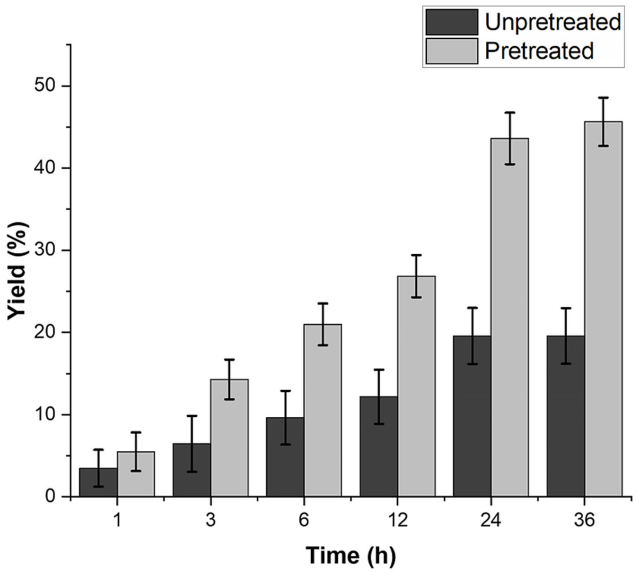

Fig. 7 Yield of reducing sugar from OPEFB hydrolysis before and after pretreatment

increases. After optimizing the pretreatment, the resulting reducing sugar yield at the end of the reaction was 45.64% which is 224% more than without pretreatment.

The reducing sugar yield from the enzymatic hydrolysis of OPEFB produced by this experiment is significantly higher compared to the studies conducted by Anita and Hidayatullah, which utilized different pretreatment combinations, resulting in yields of 34.6% [6] and 28.3% [7], respectively, under optimal conditions.

3.5 Solid characterization

The OPEFB solids analysis aims to evaluate the impact of the pretreatment on OPEFB fiber.

3.5.1 Chemical composition of OPEFB

OPEFB composition before and after pretreatment was analyzed according to the Datta approach [24] and can be seen in Table 4. OPEFB before pretreatment, contained a large amount of extractives. These extractives consist of proteins, oils, and short-chain compounds easily soluble in water. Consequently, the cellulose and lignin composition tends to increase after the pretreatment process.

In subcritical water conditions, cellulose does not dissolve into individual molecules due to its structured form (crystalline cellulose). It may dissolve as larger units in the water without undergoing a chemical breakdown (hydrolysis) into smaller components. When the temperature is increased, the degradation of cellulose will undoubtedly proceed, but this is due to the previously dissolved amorphous cellulose [38]. Therefore, subcritical water method yields cellulose-rich solids that can be converted to sugars by enzymatic hydrolysis.

In contrast, hemicellulose dropped dramatically after the subcritical water process, demonstrating that this method can degrade hemicellulose into sugar in the liquid fraction by breaking ether linkages vulnerable to acids and high temperatures [39]. Additionally, hemicellulose is more degradable than cellulose or lignin due to the amorphous structure and low degree of polymerization [40].

3.5.2 Crystallinity analysis

X-ray diffraction (XRD) examination can be used to compare the crystallinity component of OPEFB before and after pretreatment. The scattering angles (2θ) used to evaluate samples ranged from 5° to 40°. The crystallinity index (CrI) was determined using the Segal technique, as shown in Eq. 3 [41]:

$$Crystallinity\ Index\ (CrI) = \frac{I_{002} - I_{am}}{I_{002}} 100\% \quad (3)$$

Peak I_{002} is the peak with the highest peak intensity in the crystal fraction, located at 2θ = 22°, and peak I_{am} is the peak with the lowest peak intensity in the amorphous portion, located at 2θ = 18°. Fig. 8 and Table 5 provide the OPEFB's XRD spectra before and after pretreatment and its crystallinity index.

The crystallinity index of OPEFB rose following both ultrasonication and combination pretreatment. According to the table, the crystallinity index of unprocessed OPEFB is 40.52%. Following ultrasonication, the degree of crystallinity increased to 41.66. After the combined pre-treatment, the degree of crystallinity dramatically increased to 55.05%.

As demonstrated in Table 5, the rise in the crystallinity index may be related to an increase in cellulose proportion in OPEFB fiber. Lai et al. reported that the material's crystal structure is less dense as the crystallinity index increases [42]. The increase in crystal intensity might be related to the removal of both lignin and hemicellulose

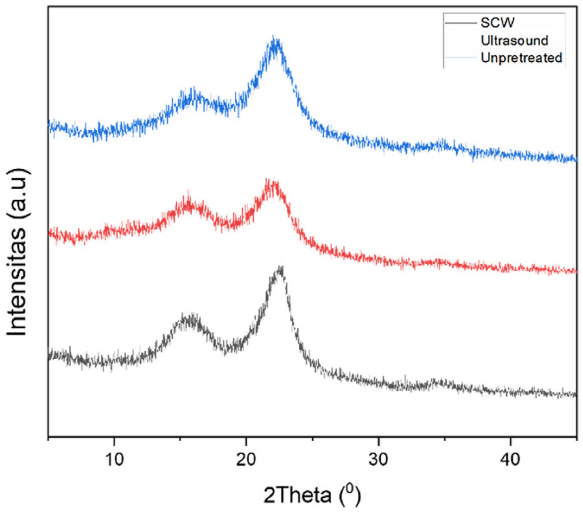

Fig. 8 XRD spectra of OPEFB before and after pretreatments

Table 5 The crystallinity of OPEFB before and after pretreatment

	CrI
Unpretreated	40.52
Ultrasound pretreated	41.66
Ultrasound-SWH pretreated	55.50

during pretreatment, which raised the relative concentration of cellulose (the only crystal in lignocellulose) [43]. Several investigations demonstrated an increase in the crystallinity index after pretreatment, which positively impacted the following treatment [36, 42, 44].

3.5.3 Functional group analysis

Fig. 9 depicts the FTIR analysis of OPEFB functional group modifications before and after pretreatments. The results indicate that the functional categories of OPEFB were relatively unchanged both before and after pretreatment.

At a wavelength of 3324 cm^{-1} (point 1), the cellulose structure is represented by hydrogen bonds (O–H) [5].

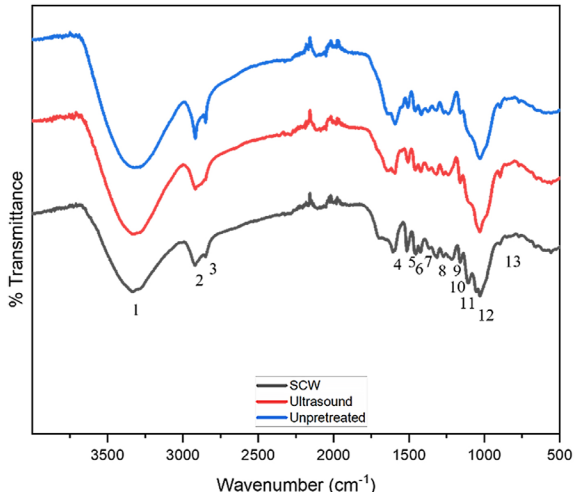

Fig. 9 OPEFB FTIR spectra before and after pretreatments

The intensities at 3331 and 3335 cm^{-1} showed an increase during the ultrasound and subcritical water preparation, aligning with the research findings that indicate an increase in cellulose content, as presented in Table 4. At point 9, which corresponds to a wavelength of 1239 cm^{-1}, the bonds in the syringil ring (lignin) and the C–O bonds in xylan (hemicellulose) exhibit a drop in intensity at a wavelength of 1217 cm^{-1} following subcritical water pretreatment [16]. The results indicate that hemicellulose and lignin are soluble in the liquid fraction. Thi et al., who investigated the effect of subcritical water on Eichhornia crassipe, reached the same conclusion [45]. Only after subcritical water treatment was the C–O group in lignin visible at a wavelength of 1108 cm^{-1}, demonstrating that subcritical water treatment can localize lignin. The summary of peak spectra in FTIR is shown in Table 6 [46].

3.5.4 Morphology analysis

Fig. 10, a scanning electron micrograph with 1000 times (lateral) and 3000 times (latitude) magnification, displays OPEFB morphological alterations before and after treatments. Additionally, Table 7 provides the average pore diameter resulting from OPEFB pre-treatment.

Table 6 Summary of FTIR peaks [46]

	Peak*				
PN*	Unp	Ult	USW	Functional Group	Band*
1	3324	3331	3335	O-H stretching of H bonds	C [5]
2	2918	2917	2919	C-H stretching of methyl or methylene	C [6]
3	2850	2850	2850	C-H stretching of methyl or methylene	C [6]
4	1592	1593	1606	C=C stretching of syringil	L [46]
5	1507	1507	1514	C=C aromatic of guaiacyl	L [46]
6	1456	1456	1456	C=C aromatic	L [42]
7	1417	1422	1424	C-H vibration	C [40]
8	1319	1317	1315	vibration C-O of syringil	L [14]
9	1239	1239	1217	Sryngil ring, C-O of xylan	L and H [42]
11	1161	1161	1161	C-O-C vibration	C and H [5]
11			1108	C-O stretching	L [46]
12	1030	1030	1030	C-O stretching	C [40]
13	896	896	896	β -glycosidic	C [5]

*Notes: Peak Number (PN); Unpretreated (Unp); Ultrasound pretreated (Ult); Ultrasound-SWH pretreated (USW); Extractives (E) ; Hemicellulose (H); Cellulose (C); Lignin (L); Ash (A)

Table 7 Average pore diameter

	Average pore diameter ($\AA$)
Unpretreated OPEFB	4.92
Ultrasonication pretreated	12.32
Ultrasound-SWH pretreated	89.05

OPEFB, which has not been processed (Fig. 10 (1A)), has a solid and sturdy surface with no fiber voids. After being treated with ultrasound (Fig. 10 (2A)), the surface of the OPEFB shows surface cracks and holes when seen at a higher magnification. Changes in solid morphology following ultrasonic pretreatment can be attributed to the loss of solvent-soluble extractives. The previous report by Park, who treated Gracilaria verrucosa to reduce sugar production, also reported that ultrasound pretreatment caused morphological damage [47]. Subcritical water pretreatment (Fig. 10 (3A)) severely damages OPEFB fibers. The resulting hole is also more extensive and more distinct. Both ultrasonic pretreatment and combination pretreatment increased pore diameter. As the exterior structure of OPEFB, the loss of hemicellulose and damage to lignin are strongly related to the degradation of OPEFB fibers. Sangian, who tested coconut coir subjected to hydrothermal

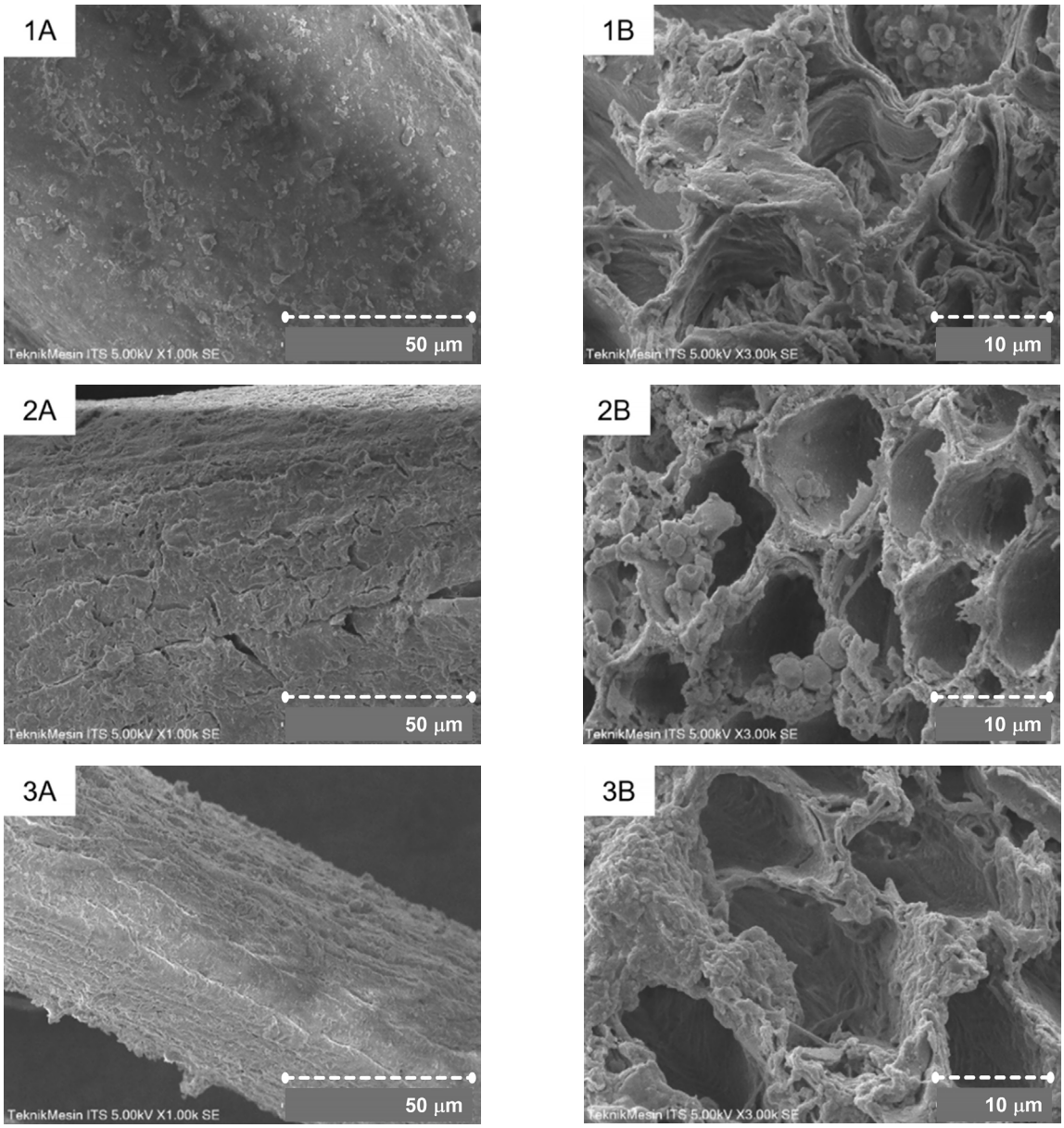

Fig. 10 SEM OPEFB magnification 1000x (A) and (B) 3000x; (1) EFB before pretreatment, (2) EFB after ultrasound pretreatment, (3) EFB after Ultrasound-SWH pretreatment

processing, reported the same thing [48]. Destruction of the structure of the biomass through physical pretreatments improves the efficiency of enzymatic hydrolysis.

4 Conclusion

Ultrasonic pretreatment is proven to increase the production of reducing sugars in subcritical water processes. At the optimum point, the reducing sugar produced increased from 3.45 g/L to 5.42 g/L. The optimum point obtained is a temperature of 208 °C, a time of 78 minutes, and a surfactant concentration of 2.6% with the expected TRS gain of 6.04 g/L. Running validation has been carried out three times with a relative error of 9.7%. Enzymatic hydrolysis after combined pretreatment increased the yield of reducing sugars up to 325% (2.84 g/L to 9.22 g/L) with an increase in yield of 224% (19.56% to 45.64%). These enhancements are attributable to the structural and chemical alterations of OPEFB, rendering it more susceptible to hydrolysis.

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