RESEARCH ARTICLE

JOURNAL OF BIOBASED CHEMICALS

Volume 1 Issue 1, 2020, pp. 48 – 59

Journal homepage: http://journal.unej.ac.id/JOBC

Optimization of Subcritical Water Assisted by Nitrogen Before Enzymatic Hydrolysis for Reducing Sugar Production

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(Submitted: 30 July 2019; Revised: 2 March 2020; Accepted: 5 June 2020)

Abstract. The objective of this study is to optimize the three significant parameters of the subcritical water (SCW) process for sugar production from coconut husk by using response surface methodology (RSM). In this study, the effect of temperature, reaction time, and solvent-feed (S/F) ratio was evaluated and discussed comprehensively. The results showed that the S/F ratio had the most significant effect on sugar yield. The optimal sugar yield was obtained at the highest S/F ratio of 23.4, the shortest time of 4.8 min, and the highest temperature of 183.6 ºC of the SCW process. The characterization results confirmed that the lignocellulose structure was changed remarkably and then contributed to the efficient processes of enzymatic hydrolysis. The parameters evaluation using RSM in this study suggests that SCW hydrolysis could be subjected to commercial purposes.

Keywords: *response surface methodology, subcritical water, enzymatic hydrolysis, sugar production, coconut husk*

1. Introduction

Biofuel from lignocellulosic biomass is considered a promising new solution for energy problems because of its cheapness, abundance, and energy security [1]. Before converting holocellulose into biofuels through biological processes, a pretreatment process is needed to destroy the complex and recalcitrant biomass structures [2]. Conventional methods using chemical solutions pose several advantages and disadvantages that affect the feasibility of industrial-scale production [3]. Although it has been proven capable of devastating the lignin

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wall to provide bacteria access to the holocellulose compound, alternative methods are still demanded due to environmental issues from the resulting waste [4]. Therefore, the attention of researchers at the moment is focused on developing sophisticated green technologies that are more sustainable to improve biomass processing.

Among the leading pretreatments, subcritical water technology, which can simultaneously function for pretreatment and hydrolysis, has received considerable attention in recent years [5]. Its rapid reaction characteristics and environmentally friendly nature make this technology the most attractive process for converting biomass. Moreover, the tunable solvent properties at different temperature ranges show that the selective optimization of hydrolysis conditions can be performed [6]. On the other hand, this technology still has weaknesses in terms of the formation of derivative products such as furfural compounds and phenolic compounds which act as inhibitors for further biological processing [7], [8]. It is considered that economically viable processes are only achieved by minimizing pretreatment costs while maximizing the yield of sugar.

Recently, we reported that the integration of subcritical water and enzymatic hydrolysis processes has successfully generated fermentable sugar from coconut husks by the severity factor approach [10]–[11]. The addition of surfactants to the subcritical water process could also increase the production of sugars [12], [13]. Kinetic studies of subcritical water hydrolysis have also been reported previously [14]. Although the work has prospered in producing biohydrogen, the yield is still relatively low due to the obtained sugar was low [11]. Therefore, optimization of the subcritical water process needs to be conducted and developed. To the best of our knowledge, the optimization of the conversion of coconut husks using subcritical water and enzymatic hydrolysis has not been reported.

To fill these research gaps, in this work, the other significant variables (solvent/feed (S/F) ratio, time, and temperature of reaction) in the SCW process have been examined and optimized by using response surface methodology to produce maximum sugar yield. Furthermore, solid characterization has been comprehensively discussed to confirm the optimization values. Subsequently, the SCW solids were subjected to enzymatic hydrolysis to get the remaining sugar. The optimization of the SCW technique before enzymatic and fermentation processes is projected making the processes more commercially viable.

2. Materials and Methods

2.1 Materials

Coconut husk was collected from Manado City, North Sulawesi, Indonesia. The preparation procedure of the sample was adapted from the previous work [9]. Some chemicals such as Dinitrosalycilic acid, commercial c*ellulase,* and *xylanase* were purchased from Sigma Aldrich, Japan.

2.2 SCW Process

The SCW equipment used in this study was modified from the preceding work [10]. The process was run by supplying the UHP Nitrogen gas (PT. Aneka Gas, Sidoarjo, Indonesia) to the reactor with a constant pressure of 80 bar. Process parameters were varied at temperatures of 130 to 170 °C for 15 to 45 min since the condition reached. The sample was washed and dried in the oven at a constant temperature of 60 °C and stored at 4 °C before being analyzed.

Central Composite Design (CCD) was applied to optimize the critical factor of the SCW process. The effect of three independent variables (Solvent/Feed (S/F) ratio, time, and temperature of reaction) at three levels toward sugar yield as the response was investigated by using Minitab 16 (Minitab Inc, ITS Surabaya, Indonesia).

2.3 Enzymatic Hydrolysis

The experimental and analytical procedures for enzymatic hydrolysis were conducted based on previous work [12]. All of the coconut husk solid was hydrolyzed using a combination of commercial *cellulase* and *xylanase*. The hydrolysis process was carried out in a shaker incubator and operated at 60 °C and 125 rpm. Sugar obtained during the process was analyzed every 8 h for 48 h.

2.4 Analytical Method

The reducing sugar was obtained following SCW and enzymatic hydrolysis process. It was measured by using the dinitrosalicylic acid (DNS) method [15]. Hydrolysis data were analyzed by applying the two-way analysis of variance (ANOVA). Data are presented as mean standard deviation based on triplicate analyses. Pretreated and unpretreated samples were examined by using Scanning Electron Microscopy (SEM) Evo MA 19 (Carl Zeiss, England), X-ray diffraction (XRD) X'Pert PRO (PANalytical B.V, Holland), and Fourier Transform Infrared (FTIR) Nicolet iS10 spectrophotometer.

3. Results and Discussions

3.1 Response Surface of SCW process

In this work, three critical parameters of the SCW process were evaluated to determine the variable that has a significant effect in reducing the concentration of sugar using response surface methodology (RSM). Table 1 shows the design and result of the three parameters in the SCW process using a central composite design (CCD). As shown in Table 1, the yields obtained from the SCW pretreatment process varied from 3.37% to 9.89%. The highest reducing sugar yield of 9.89% was attained under a reaction condition of 170 °C for 15 min and the S/F ratio of 20.

Table 1. Central composite design matrix along with predicted and experimental values for reducing sugar yield in the SCW process

Runs	Reaction time (min)	S/F ratio	Reaction temperature (°C)	Sugar concentration (g/L)	Reducing sugar yield (%)	
					Observed	Predicted
1	30	15	150	4.795	4.295	4.255
2	30	23.4	150	4.026	4.026	4.426
3	30	15	150	6.499	4.299	4.255
4	15	20	130	3.589	3.589	3.219
5	30	15	150	4.841	4.241	4.255
6	15	10	130	3.423	3.423	3.280
7	15	20	170	4.946	4.946	4.802
8	30	15	150	3.363	4.263	4.255
9	45	10	170	7.585	7.585	7.474
10	30	6.6	150	6.379	7.379	7.697
11	45	10	130	6.711	6.982	6.642
12	45	20	170	3.725	3.981	3.641
13	45	20	130	3.061	3.016	2.834
14	55.2	15	150	3.649	4.078	4.451
15	30	15	150	2.609	4.258	4.255
16	4.8	15	150	2.247	2.247	2.602
17	30	15	116.4	2.564	2.729	3.129
18	30	15	183.6	4.177	4.830	5.158
19	15	10	170	5.188	5.188	4.888
20	30	15	150	3.544	4.244	4.255

The experiment data of sugar yield was used to determine the coefficient of the regression equation (Eq. 1) where X_1, X_2, and X_3 are reaction time, S/F ratio, and temperature. The second-order polynomial equation can be used to predict the yield of reducing sugar.

$$\begin{aligned} Yield = -20.4 + 0.513X_1 - 0.07X_2 + 0.192X_3 - 0.00189X_1X_1 + 0.0168X_2X_2 \\ - 0.0005X_3X_3 - 0.01531X_1X_2 - 0.00095X_1X_3 + 0.00175X_2X_3 \end{aligned} \tag{1}$$

In the general SCW Process, the temperature is one of the significant parameters toward cracking and hydrolysis performance of lignocellulose. It is due to the properties of SCW which help in the hydrolysis process (i.e., dielectric constant, density, viscosity, etc.) is a function of the temperature [16]. Figure 1 depicts a 3-D plot of the response surface for the reducing sugar yield. As shown in Figure 1, by increasing temperature, reducing sugar production was increased. The degradation of hemicellulose and cellulose occurred at high temperatures because water was more reactive to breaking down the complex carbohydrate molecules. Hydrolysis biomass runs slowly at a temperature below 100 °C. Prado et al. (2016) revealed that free sugars and most hemicelluloses reacted above 170 °C. Muharja et al. (2017) showed the same result that hemicellulose extract from coconut husk increased from 67.8% to 73.94% with increasing temperature from 150 to 160 °C.

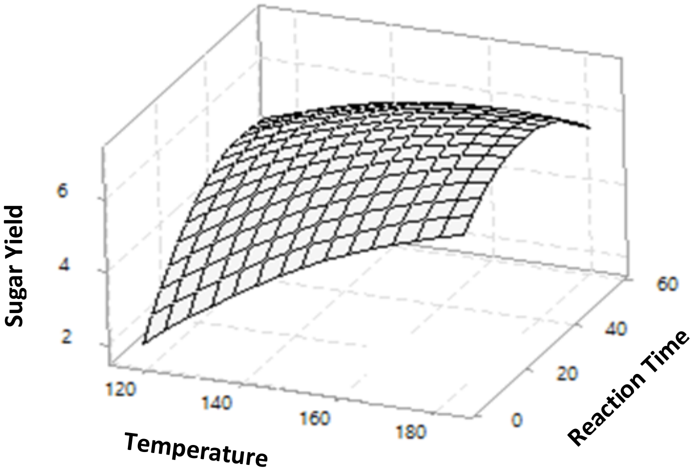

Figure 1. Surface response from RSM design

Another critical parameter of hydrolysis using SCW is reaction time. As shown in Figure 1, the yield of reducing sugar increased with increasing time of reaction and then decreased. This can be explained by the fact that the contact between the solvent and the substrate causes the carbohydrate complex molecules to be increasingly hydrolyzed. This result is similar to the study of cellulose recovery from water hyacinth by Thi et al. (2017). They revealed that reducing sugar increased at 15 to 35 min and then decreased.

Table 2. Analysis of variance (ANOVA) of model parameters

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Model	9	48.6301	5.4033	1.86	0.173
Linear	3	30.8721	10.2907	3.55	0.056
Temperature	1	8.4775	8.4775	2.92	0.118
Reaction Time	1	2.3972	2.3972	0.83	0.385
S/F Ratio	1	19.9974	19.9974	6.89	**0.025**

Source	DF	Adj. SS	Adj. MS	F-Value	P-Value
Square	3	6.3153	2.1051	0.73	0.560
Temperature*Temperature	1	0.5708	0.5708	0.20	0.667
Time*Time	1	2.6065	2.6065	0.90	0.366
S/F Ratio*S/F Ratio	1	2.5600	2.5600	0.88	0.370
2-Way Interaction	3	11.4426	3.8142	1.31	0.323
Temperature* Time	1	0.6441	0.6441	0.22	0.648
Temperature*S/F Ratio	1	0.2415	0.2415	0.08	0.779
Time*S/F Ratio	1	10.5570	10.5570	3.64	0.086
Error	10	29.0098	2.9010		
Lack-of-Fit	5	7.2083	1.4417	0.33	**0.875**
Pure Error	5	21.8015	4.3603		
Total	19	77.6399			

Note. The bold letter denoted the significant value (at a confidence level of 95%).
DF, Adj. SS, and Adj. MS means Degree of Freedom, Adjusted Sum of Squares, and Adjusted Mean of Squares, respectively.

The amount of water affected sugar yield. In this study, the S/F ratio is the most significant parameter in the hydrolysis process (see Table 2). As shown in Figure 2, hydrolysis of reducing sugar increased with increasing S/F ratio. Based on the result of this study, more solvents used in the process cause complex carbohydrate molecules to be more easily hydrolyzed. A higher S/F ratio caused the solution in the reactor to be not easily saturated. In the batch process, the saturation of the solution becomes very important because it affects the solubility of the hydrolyzed sugar. The same result was reviewed by Prado et al., (2016) where sugar yield increased with increasing S/F for batch SCW system.

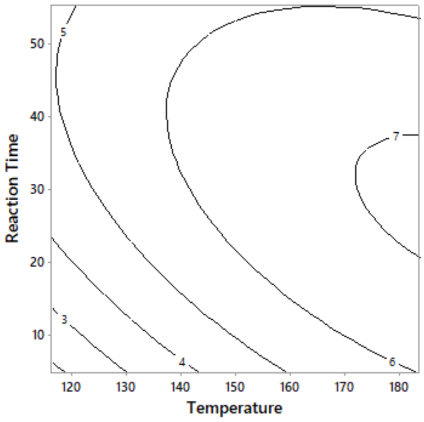

Figure 2. Contour response from RSM Design

3.2 Statistical Analysis and Model Fitting

P-value is a parameter to determine the significant factor of the experiment. A p-value of less than 0.05 indicates factors that are significant at the probability level of 95%. Table 2 is an analysis of variance (ANOVA) to fit the models. From Table 2, the linear coefficient (reaction time, temperature), square coefficient, and cross coefficient were not significant. Surprisingly, the S/F ratio is a highly influential parameter in reducing sugar yield through SCW. The lack-of-fit test shows that the CCD model is sufficiently precise for predicting reducing sugar yield by SCW hydrolysis. Based on the model, the operating conditions for the optimum reducing sugar yield were on the temperature reaction 183.6 ° C with the S/F ratio of 23.4 for 4.8 min.

3.3 Enzymatic Hydrolysis

Figure 3 shows the time courses of the hydrolysis process following SCW employed. The sugar concentration increased significantly from 0 to 8 h of hydrolysis time. This phenomenon may be due to enzymes having hydrolyzed cellulose and hemicellulose entirely during that condition for an initial 8 h. A study by Sánchez-Ramírez et al., (2017) revealed that after incubation at 60 ° C, enzyme activity diminished continuously and reached 20% of its initial activity after 4 h of hydrolysis. As shown in Figure 3, the highest yield of reducing sugar obtained after enzymatic hydrolysis for 48 h was 2.58 g/L. Using two-way ANOVA, it is known that the SCW process has a significant effect on the concentration of sugar compared to the hydrolysis of untreated solids.

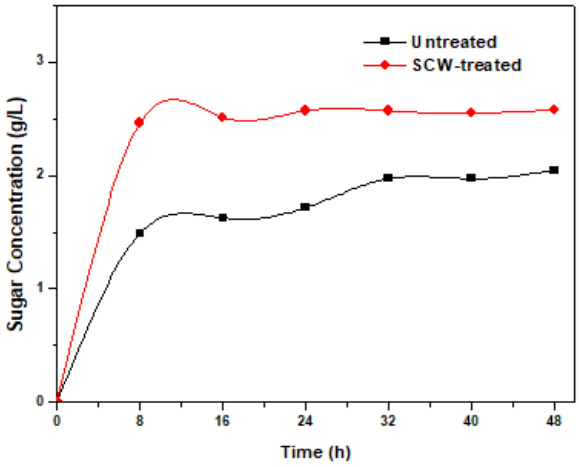

Figure 3. Time courses of the sugar concentration during enzymatic hydrolysis of (a) untreated and (b) SCW-treated solid at optimum conditions

3.4 Characteristics of solid residue

Figure 4 shows the comparison of diffraction patterns between untreated and SCW-treated coconut husks at optimum conditions. XRD was used to detect CrI values from coconut husk. The value CrI of coconut husk before and after pretreatment was 14.69% and 8.33%, respectively. The SCW-treated coconut husk showed a broad diffraction pattern with higher intensity compared to the untreated coconut husk. Ciftci and Saldana (2015) reported that the crystalline index of sweet blue lupin increased from 11.5% to 58.6% after SCW pretreatment. Mohan et al. (2015) also reported the crystalline index of the sample increased from 50.55% to 65.83% after pretreatment. This can be explained by the degradation of amorphous lignin and hemicellulose after SCW pretreatment, leading to an increase in the amount of crystalline part from cellulose-enriched residue.

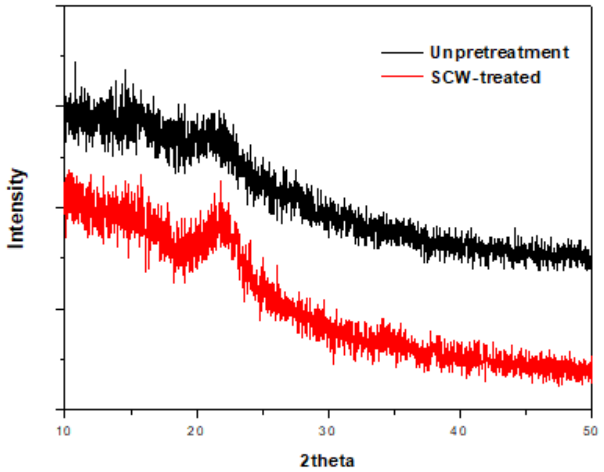

Figure 4. XRD pattern of (a) untreated and (b) SCW treated solid at optimum conditions

Fourier transform infrared spectroscopy (FTIR) was used to identify and characterize the treated coconut husk solid after SCW pretreatment. The FTIR spectra of untreated and SCW-treated samples are shown in Figure 5. The emergence of the peak at the wavelength of 1300 indicates the attenuation of the OH bond between the cellulose. A vibration of aromatic rings indicates a peak of 1600. The range is 1030 indicates the stretching of cellulose and hemicellulose in C-O. The emergence of vibration peaks that exist in pretreatment samples indicates the process of lignification and dissolution of hemicellulose and cellulose.

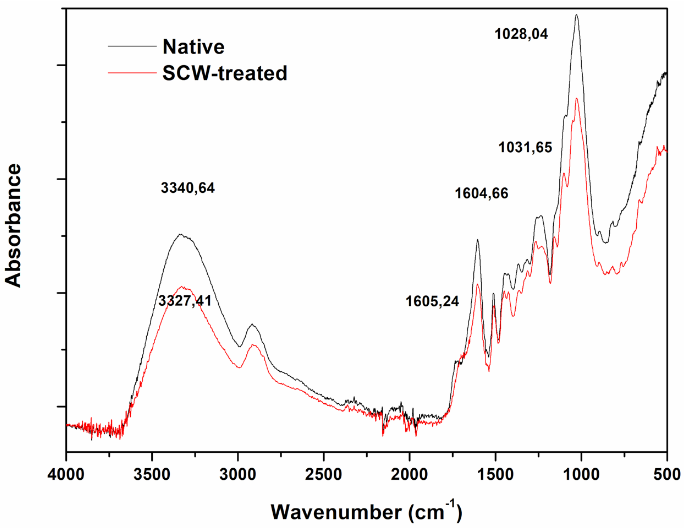

Figure 5. FTIR spectra of (a) untreated and (b) SCW-treated solid at optimum conditions

The surface morphology of untreated and SCW-treated solid was examined using SEM. Figure 6 shows the SEM images of (a) untreated and (b) SCW-treated solids at optimum conditions. The surface of coconut husk before SCW pretreatment is marked by some boundary edges and does not show the presence of any pores, trenches, or surface cracks, while cracks and trenches can be seen on the surface of the SCW-treated solid. These images of microscopic structure differences demonstrate that the SCW pretreatment could efficiently disintegrate the lignocellulose cell wall, resulting in exposure of internal structure [20]. These crystal structural changes have been managed to expose cellulose to enzyme access and increase the digestibility of enzymatic hydrolysis [22].

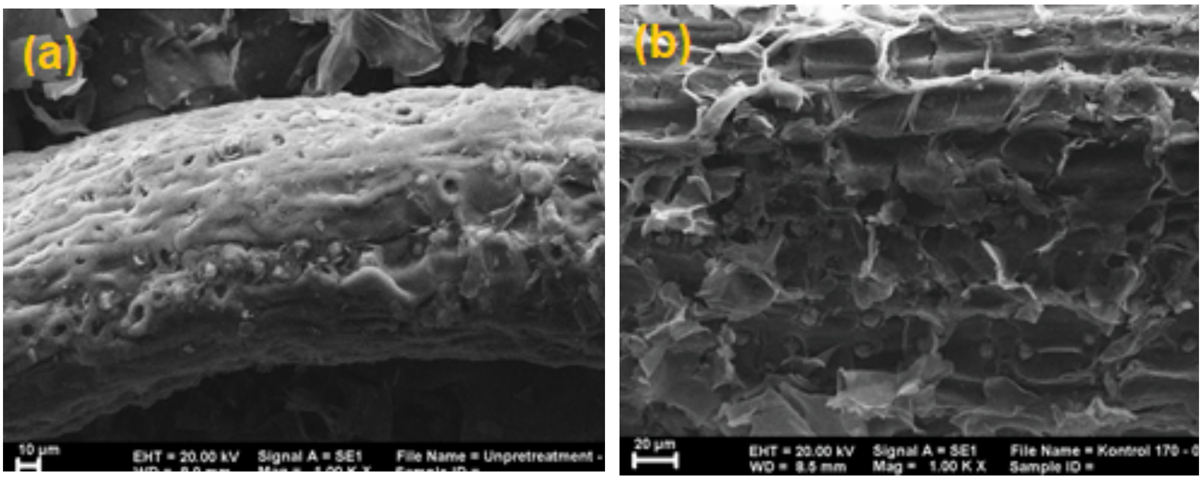

Figure 6. The morphological structure of (a) untreated and (b) SCW-treated solid at optimum conditions

4. Conclusion

Reducing sugar has been successfully gained from coconut husk using a combination of SCW and enzymatic hydrolysis. The highest yield of sugar was 9.89% with a sugar concentration of 4.946 g/L. The variable water-solid ratio has a significant effect, where the high ratio will produce a high yield. From the optimization, the concentration of reducing sugar from the RSM model was 183.6 °C for 4.8 min and 23.4 S/F ratio

ACKNOWLEDGMENT

This research was supported by funding from the Directorate General of Resources for Science, Technology, and Higher Education, Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

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