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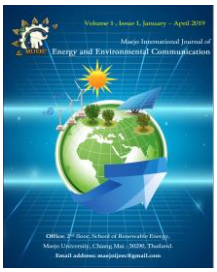

ARTICLE

Development of a novel sequential pretreatment strategy for the production of bioethanol from coconut pulp residue

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ABSTRACT

Coconut pulp residues waste generated after extraction of milk or oil. These wastes end up as feed to animals, fertilizers and firewood/cooking fuel whilst large quantities often left to rot in the field, which causes cause pollution, waste disposal problems and increase handling cost for farmers. In order to alleviate this problem, coconut pulp residue was used as feedstock for bioethanol production. However, improvements on pretreatment are necessary to produce higher sugar concentration prior to fermentation. Bioethanol production from coconut pure pulp residue (PPR) and combined pulp residue (CPRS) was investigated. The results showing 40 minutes' pre-hydrothermal treatment time and 2% mild sulphuric acid for PPR and 20 minutes' hydrothermal treatment time and 2% mild sulphuric acid for CPRS.

1. Introduction

Worldwide production of biofuel has been increasing at a remarkable pace. It is derived from the impending concern of fuel sustainability and due to the environmental problems related to the utilization of non-renewable energy (Manmai et al., 2017a,b,c ; Manmai et al., 2018a,b). Among the different types of biofuels, bioethanol is one of the prominent as its characteristics similar to gasoline. Bioethanol can be produced in an array of renewable sources (Manmai et al., 2019a,b,c; Manmai et al., 2020). Although there have been several biomasses being used for bioethanol production, it still requires new techniques and biomasses that can improve production and sustain the demand in bioethanol (Mariano et al., 2018). The material used in this study is beneficial, especially to countries with massive production of coconut. Along with the processing of coconut for extraction of milk or oil, it generates a huge amount of pulp residue which is considered as waste.

Normally, these pulp residues are being used as fertilizer and feed for livestock feeding. However, enormous quantities are left to rot in the field. Therefore, instead of ending up as waste, coconut pulp residue was used for bioethanol production. The techniques especially the sequential pretreatment and hydrolysis applied to pulp residue are simple and can be executed easily as it is believed to be applicable to several similar lignocellulosic biomasses as it also not require sophisticated and/or special equipment. Besides, the results also showed the efficiency of the techniques as it caused high production of sugars and most especially bioethanol when compared to others. Moreover, the paper clearly demonstrates a way of creating an economically valuable product from waste. In concerns pertaining to the sustainability of biomass, coconut fruits all year round unlike

most of the other fruit trees that only produced their fruit during a certain season. Consequently, processing of coconut also operates all year round; hence; there is also a continuous generation of waste pulp residue. Therefore, large production of coconut pulp residue can suffice the needs for bioethanol production. The material used in this study is beneficial, especially to countries with massive production of coconut. Along with the processing of coconut for extraction of milk or oil, it generates a huge amount of pulp residue which is considered as waste.

Normally, these pulp residues are being used as fertilizer and feed for cows. However, enormous quantities are left to rot in the field. We were able to produce bioethanol by adopting conventional method of hydrothermal/mild acid pretreat-ment, neutralization, filtration, and aging with minor alterations in both pretreatments process from the industrial waste biomass of coconut pulp residue. Therefore, in this study instead of ending up as waste, coconut pulp residue was used for bioethanol production.

2. Materials and methods

2.1 Material preparation

Coconut pulp residue was obtained from a local oil processing company in Chiang Mai, Thailand. The coconut oil extraction involves two (2) sets of processing which resulted to two types of coconut pulp residue: pure pulp residue (PPR) and pulp resdue and seed coat (CPRS) as shown in Figure 1.

Figure 1. Coconut pulp residues [A] Pure pulp residue [B] Combined pulp residue and seed

The biomasses are dried for 48 hours dried in an economy laboratory oven (Binder ED-115, Germany) and and kept in plastic bags at room temperature until further use. All chemicals analytical chemicals were obtained from Sigma Aldrich USA or Merck, Germany. Commercial enzyme used in this study was purchased Union Science Company, Chiang Mai Thailand.

2.2.2 Optimization of pretreatment using central composite design (CCD)

The interaction between the pre-hydrothermal treatment time and acid concentration on the release of sugars can easily be determined using response surface methodology. The hydrothermal heating time (0, 20, 40 and 60 minutes) and H_2SO_4 (1%, 2%, 3%) concentration were chosen as independent variables and the amount of total and reducing sugars was used as dependent variables and analyzed using central composite design. The central composite design is used in optimization of analytical procedures and has three (3) parts: (1) full factorial design (2) star design (3) cental point. This type of experimental design is limited into 2 factors as higher factors causes inefficiency in the modelling of quadratic functions. Presented in Figure 7 are the three level factorial designs (Khuri and Mukhopadhyay, 2010). In this design, there are three experimental levels: − 1, 0, 1 as presented in Table 1. Total runs for the response surface optimization were 16 and three replicates for each run were employed to verify any changes in the estimation and experimental procedure.

Table 1. Sugar concentration parameters and their values used for the experiment

Factors	Unit	-1	0	1
Hydrothermal pretreatment time	Minutes (mins)	20	40	60
Acid concentration	Percent (%)	1	2	3

2.3 Sequential Pretreatment

2.3.1 Hydrothermal Pretreatment

The method presented by Amadi et al. (2018) was used in hydrothermal pretreatment with minor modifications. Dried coconut pulp residues (PPR and CPRS) were prepared and submerged into 200 ml of distilled water in 250 ml Erlenmeyer flask. The flask was then placed in a hot plate (HTS-1003, Japan) and boiled with different heating times of 20, 40 and 60 minutes. After completion, the solution was filtered using a cheesecloth to separate the solids from the liquid. The liquid hydrolysate recovered was kept at 4 °C for further use and the solid residues proceeded to acid pretreatment.

2.3.2 Acid pretreatment

The residual solids from hydrothermal pretreatment were further hydrolyzed using mild acid. The modified method by Sritrakul et al. (2017) was applied to the pretreated coconut pulp residues. The solids from pretreatment 1 was placed in a flask and added with different concentrations of H_2SO_4 (1%, 2%, 3%), respectively. The flask were then capped with cotton plug and positioned in vertical autoclave for 20 minutes at 121 °C. The solutions were then cooled to room temperature before enzymatic hydrolysis.

2.4 Enzymatic hydrolysis

The enzymatic hydrolysis was carried out with commercial cellulase enzyme with 2398 units/g and β-glucosidase 577 units/g from Union Science Company, Chiang Mai, Thailand. The acid pretreated coconut pulp was transferred in a beaker and the hydrolysate from hydrothermal pretreatment was added separately. The pH of the solution were adjusted to 5 and 2% (v/v) of cellulase was added. The mixture of was kept in was incubated at 50°C for 24 hours. The hydrolysate was filtered and analyzed for total and reducing sugars (Bautista et al., 2018; Casabar et al., 2019).

2.5 Sugar Analysis

The total and reducing sugar content of the PPR and CPRS hydrolysate was measured using the phenol-sulfuric acid and DNS method. The phenol-sulfuric acid method was carried out with diluted 0.5 ml of sample combined with 0.5 ml of 5% phenol and 2.5 ml of 98% sulphuric acid (Dubois et al., 1956). The solution was kept for 10 minutes and the absorbance was read using the spectrophotometer (Drawell-DV-8000, China) at 490 nm. Reducing sugar was estimated by diluted 0.5 ml of sample and added with 0.5 ml of f 3,5-dinitrosalicylic acid (DNS) solution and heated in a boiling water bath for 15 minutes followed by addition of 4 ml of distilled water (Miller, 1959). The mixture was then cooled to room temperature and the absorbance was measured at 540 nm using spectrophotomter. Glucose was used as standard.

Soybean oil used in this study as a feedstock was purchased at the nearby supermarket in Kuantan, Pahang. The banana peels and eggshell were collected from nearby stalls and restaurant in Kuantan, Pahang before used as a catalyst in the production of methyl ester. An analytical grade of methanol, n-hexane, n-heptane, petroleum ether, and chloroform were obtained from Merck. An analytical grade of methyl heptadecanoate, phenolphthalein, 4-nitroaniline and 2,4-dinitroaniline were purchased from Sigma Aldrich company (Switzerland).

Nomenclature and Abbreviation

K_2CO_3	Potassium carbonate
C_aO	Calcium oxide
FT-IR	Fourier transform infrared spectrometer
XRD	X-ray diffractometry
SEM	Scanning electron microscopy

2.1 Preparation of Catalyst

The collected waste of banana peels and eggshell were thoroughly washed with tap water to remove dirt and organic matter. The banana peels were dried in the oven about 80 h at temperature 100°C while the eggshell was dried for 24 h at a temperature of 105°C. Then, the dried banana peels and eggshell were powdered separately using a grinder. The banana peels powder was calcined in a furnace at 700 °C for 4 h and the eggshell powder was calcined at 900 °C for 3 h. The calcined banana peels and eggshell were assigned as CBP and CES respectively. The CES was impregnated to CBP with different concentrations (10 wt. %, 20 wt. %, 30 wt. %, and 40 wt. % in order to get the desired catalyst, $CaO\text{-}K_2CO_3$. The $CaO\text{-}K_2CO_3$ catalyst was analyzed via Fourier Transform Infrared (FTIR) spectrophotometer, Brunauer-Emmett-Teller (BET) analysis, Thermogravimetric analysis (TGA) and Field Emission Scanning Electron Microscopy (FE-SEM). The basic strength of the catalyst was tested using Hammett indicators; phenolphthalein (H_ = 8.2), 2, 4-dinitroaniline (H_= 5), 4-nitroaniline (H_=8.4).

2.2 Reaction process

The transesterification of soybean oil and methanol were carried out in a 50 ml one-neck round bottom flask with a magnetic stirrer, condenser, and thermometer, immersing in a water bath. The reaction process was controlled at a temperature of 65 ± 2 °C. Several reaction parameters (catalyst amount, reaction hours and the molar ratio of methanol to oil) were studied to identify the optimum reaction conditions needed. The product solution was allowed to cool so that the glycerol can be separated by gravity. Centrifugation was used to further separate the products and by product layers to obtain pure methyl esters.

2.3 Gas chromatography analysis

The GC-FID with a capillary column of HP-INNOWAX (length 30 m x internal diameter 0.25 mm x film thickness 0.25 μm) was used to determine the conversion of methyl ester produced. Methyl heptadecanoate was used as an internal standard. The methyl ester conversion was determined following the European regulation procedure EN 14103 by comparing the identified methyl ester peaks with respective internal standard.

3. Results and discussion

Optimization of enzymatic hydrolysis through response surface methodology (RSM)

Coconut pulp residues composed of 36.1% of cellulose, 44.1% hemicellulose and 16.3% of lignin according to (Sangkharak et al., 2019). These cellulosic materials are very attractive as a feedstock for generation of bioproducts (Cybulska et al., 2014). However, these recalcitrant fractions are required to partially disrupt, allowing an increase of the surface area and enhancing the accessibility for the enzymatic attack in the hydrolysis (Batista et al., 2019). To meet this

challenge, novel lignocellulosic fractionation technologies to separately catalytic conversion cellulose to C5 and C6-sugars are presented in a number of literature but improvements are necessary due to technical and economic limitations. Hydrothermal pretreatment is one of the most effective pretreatment to different types of biomass (Zakaria et al., 2015). The advantage of hydrothermal pretreatment is that it only uses water as a catalyst. The hydronium ion present from the water acts as acid catalyst and hydrolyzes hemicelluloses polysaccharides to monomers and oligomers (Jeong and Lee, 2015). It was also proven effective and cost-efficient in a wide variety of biomasses such as rice and wheat straw, corn and sugarcane (Chen et al., 2011; Esteghlalian et al., 1997; Hsu et al., 2010; Saha et al., 2013). Additionally, it only produces low concentration of sugar degradation products such as furfural and 5-hydroxymethylfurfural which does not affect enzymatic hydrolysis. The hemicelluloses in hydrothermal pretreatment, however, does not completely solubilized and lignin is removed to a limited extent only in the biomass (Kumar et al., 2009). Thus succeeding pretreatment is necessary. Acid pretreatment, meanwhile, employs acid as catalysts that have stronger effect on hemicellulose and lignin (Tomás-Pejó et al., 2011). It involves the use of concentrated and dilute acids to break the rigid structure of the lignocellulosic biomass (de Jong and Gosselink, 2014). Various acids have been reported utilized, such as sulfuric acid, nitric acid, hydrochloric acid, phosphoric acid, peracetic acid and oxalic acid. Consequently, dilute sulfuric acid is the most widely exploited due to low cost, high reactivity and develops only a little or no inhibitors that affect the saccharification stage of bioethanol production compared to concentrated acids (Singh et al., 2015). One of the main advantages of acid pretreatment over other chemical pretreatment (i.e. alkaline) is that it is capable of hydrolyzing the hemicellulose into fermentable sugars. Therefore, sequential hydrothermal and acid pretreatment followed by enzymatic hydrolysis are applied to the coconut pulp residues for saccharification. The effect of pre-hydrothermal and post-acid treatment was optimized using response surface for the efficient saccharification of PPR and CPRS. The hydrothermal pretreatment residence time (0, 20 mins, 40 mins and 60 mins) and the post-acid concentrations (1%, 2%, 3%) were chosen as independent variables and the amount of total and reducing sugars was used as dependent variables for optimization. The resulting total and reducing sugar of PPR and CPRS were fitted to second-order model for the determination of optimum conditions.

Equations 1 and 2 present the coefficient of a linear term A (Hydrothermal pretreatment time) and B (H_2SO_4 concentration).

$$TS=244.82+62.74A+12.14B+0.7866AB-119.42A^2+1.77B^2 \quad (1)$$

$$RS=133.35+70.97A-2.99B-6.24AB-27.41A^2+10.36B^2 \quad (2)$$

Whereas, TS and RS stands for total sugar (g/L) and reducing sugar (g/L) from pure pulp residue, respectively; A and B are hydrothermal condition (mins) and H_2SO_4 concentration (%), respectively.

The result of parameter estimates and analysis of variance (ANOVA) presented in Table 2. P-values less than 0.5000 indicate the model terms are significant, indicating that all independent variables had a significant effect on sugar yield of PPR treated sequentially with hydrothermal and acid pretreatment. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant.

Table 2. Parameter estimates and analysis of variance (ANOVA) for production of TS and RS from hydrothermal and post-steam-acid pretreatment of PPR

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Total Sugar						
Model	1.360E+05	5	27196.78	3231.06	< 0.0001	significant
A-Hydrothermal pretreatment	50875.75	1	50875.75	6044.19	< 0.0001	
B-Acid concentration	2609.25	1	2609.25	309.99	< 0.0001	
AB	7.54	1	7.54	0.8952	0.3566	
A^2	59507.58	1	59507.58	7069.67	< 0.0001	
B^2	11.70	1	11.70	1.39	0.2538	
Residual	151.51	18	8.42			
Lack of Fit	20.10	2	10.05	1.22	0.3202	not significant
Pure Error	131.41	16	8.21			
Cor Total	1.361E+05	23				
Reducing Sugar						
Model	68577.38	5	13715.48	10753.24	< 0.0001	significant
A-Hydrothermal pretreatment	50853.39	1	50853.39	39870.20	< 0.0001	
B-Acid concentration	105.49	1	105.49	82.71	< 0.0001	
AB	256.09	1	256.09	200.78	< 0.0001	
A^2	2483.56	1	2483.56	1947.17	< 0.0001	
B^2	392.72	1	392.72	307.90	< 0.0001	
Residual	19.13	15	1.28			
Lack of Fit	0.8736	1	0.8736	0.6698	0.4268	not significant
Pure Error	18.26	14	1.30			
Cor Total	68596.52	20				

In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The predicted R^2 of total and reducing sugar meanwhile are 0.9982 and 0.9995, which is in reasonable agreement with the adjusted R^2 of 0.9986 and 0.9996, reflecting high significance of the model. Additionally, the adequacy precision was 97.30 for total sugar and 80.01 for reducing sugar, showing that the models can be

used to predict the optimal condition for the sequential hydrothermal and post-oven-acid assisted treatment of PPR. Finally, the lack of fit of the models was 0.32 (TS) and 0.43 (RS), proves that the model is significant and that the experimental data fitted the model.

The effects of pre-hydrothermal treatment post-steam-acid treatment on total and reducing sugars are shown in Figure 2. The total sugar concentrations were increasing from 20 minutes to 40 minutes pre-hydrothermal treatment time but declined on 60 minutes. Inverse reaction was observed meanwhile on acid concentration, total sugar are at peak at lower (1%) and higher (3%) concentration but reduced in middle (2%). Consequently, reducing sugar has increasing concentration based on increasing pre-hydrothermal treatment time. However, in acid post-treatment effect, reducing sugars favours lower concentration. Correlating the total and reducing sugars, the maximum values obtained 252.46 g/L and 153.97 g/L, respectively. The values were achieved in the optimal conditions of 40 minutes pre-hydrothermal treatment and post-steam-acid treatment of 2% H_2SO_4.

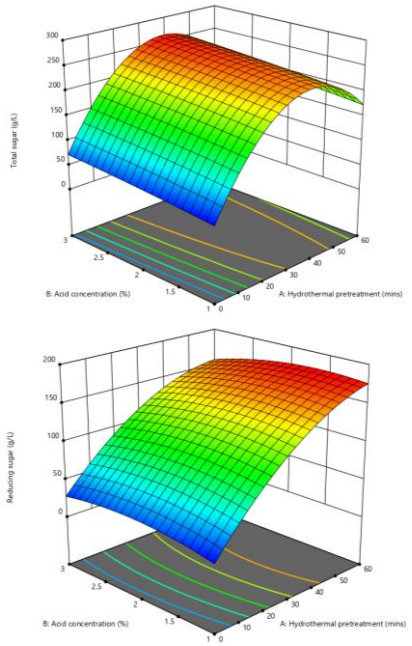

Figure 2. Three-dimensional (3D) response surface plots of independent variables: TS and RS from hydrothermal and post-steam-acid pretreatment of PPR

The effects of pre-hydrothermal treatment post-steam-acid treatment on total and reducing sugars are shown in Figure 2. The total sugar concentrations were increasing from 20 minutes to 40 minutes pre-hydrothermal treatment time but declined on 60 minutes. Inverse reaction was observed meanwhile on acid concentration, total sugar are at peak at lower (1%) and higher (3%) concentration but reduced in middle (2%). Consequently, reducing sugar has increasing concentration based on increasing pre-hydrothermal treatment time. However, in acid post-treatment effect, reducing sugars favours lower concentration. Correlating the total and reducing sugars, the maximum values obtained 252.46 g/L and 153.97 g/L, respectively. The values were achieved in the optimal conditions of 40 minutes pre-hydrothermal treatment and post-steam-acid treatment of 2% H_2SO_4.

For the CPRS, however, the optimization equations from response surface methodology are shown in equation 3 and 4:

$$TS=297.69+69.35A+28.39B+20.72AB-171.22A^2+6.11B^2 \quad (3)$$

$$RS=241.31+67.10A+17.20B+10.30AB-124.32A^2-22.3B^2 \quad (4)$$

Whereas, TS and RS stands for total sugar (g/L) and reducing sugar (g/L) from pure pulp residue, respectively; A and B are hydrothermal condition (mins) and alkali concentration (%), respectively.

The ANOVA test results are in Table 3. The model found to be significant; therefore, independent variables affect the total and reducing sugar of CPRS. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The value of predicted R2 of total and reducing sugar of 0.9962 and 0.9958 are in reasonable agreement with the adjusted R2 of 0.9972 and 0.9971, which demonstrates high significance of the models. Additionally, the adequacy precision was 88.38 for total sugar and 88.66 for reducing sugar showing that the models can be used to predict the optimal condition for the sequential hydrothermal and post-steam-acid assisted pretreatment of CPRS. The lack of fit of the models was 2.61 (TS) and 2.59 (RS), proves that the model is significant and that the experimental data fitted the model.

Table 3 Parameter estimates and analysis of variance (ANOVA) for production of TS and RS from hydrothermal and post-steam-acid pretreatment of CPRS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Total Sugar						
Model	1.479E+05	5	29571.47	1338.14	< 0.0001	significant
A-Hydrothermal pretreatment	55840.85	1	55840.85	2526.86	< 0.0001	
B-Acid concentration	11270.57	1	11270.57	510.01	< 0.0001	
AB	3845.65	1	3845.65	174.02	< 0.0001	
A^2	1.055E+05	1	1.055E+05	4775.56	< 0.0001	
B^2	136.62	1	136.62	6.18	0.0261	
Residual	309.38	14	22.10			

Lack of Fit	128.79	3	42.93	2.61	0.1037	not significant
Pure Error	180.59	11	16.42			
Cor Total	1.482E+05	19				
Reducing Sugar						
Model	94835.68	5	18967.14	1313.07	< 0.0001	significant
A-Hydrothermal pretreatment	51368.07	1	51368.07	3556.14	< 0.0001	
B-Acid concentration	4376.46	1	4376.46	302.98	< 0.0001	
AB	1192.77	1	1192.77	82.57	< 0.0001	
A^2	42775.27	1	42775.27	2961.27	< 0.0001	
B^2	1676.80	1	1676.80	116.08	< 0.0001	
Residual	202.23	14	14.44			
Lack of Fit	60.94	2	30.47	2.59	0.1163	not significant
Pure Error	141.29	12	11.77			
Cor Total	95037.91	19				

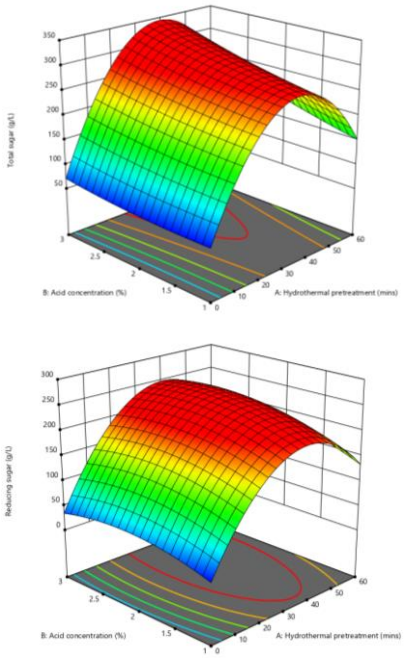

Figure 3. Three-dimensional (3D) response surface plots of independent variables: TS and RS from hydrothermal and post-steam-acid pretreatment of CPRS

In CPRS, the total sugar are affected negatively by longer pre-hydrothermal treatment time. However, it was positively affected by increasing acid concentration as depicted in Figure 3. On the other hand, reducing sugars or the fermentable sugars on CPRS, are at highest concentration on shorter pre-hydrothermal treatment time which agrees with the total sugar. The acid concentration however favours 2% H_2SO_4 concentration. As a result, the maximal sugars achieved for total and reducing sugars are 253.71 g/L and 207.32, respectively. The optimal conditions are 20 minutes pre-hydrothermal treatment and 2% H_2SO_4 in post-steam-acid pretreatment.

3.1 Comparison of Pure Pulp Residue (PPR) and Combined Pulp Residue and Seed Coat (CPRS)

The contour plots of PPR and CPRS sequentially treated with hydrothermal and acid pretreatment as presented in Figure 1 and 2 are as follows: The optimal conditions for hyrothermal pretreatment of PPR and CPRS are 40 minutes, 20 minutes, and H_2SO_4 concentration of 2% for post-acid treatment for both. The maximal total and reducing sugars achieved from the optimal condition of PPR was 252.46 g/L (0.51 g/g) and 153.97 g/L (0.31 g/g) while in CPRS 253.71 g/L (0.51 g/g) and 207.32 g/L (0.42 g/g). Evidently, CPRS has significantly higher sugar concentration compared to PPR. When further analyzed, the degree of polymerization (DP) of CPRS was calculated to be 1.20, which is lower with PPR of 1.67. Casabar et al. (2019) mentioned the importance of calculation of DP; whereas it represents the number of monomeric sugars in macromolecule. It is essential that the DP is low since higher DP would eventually require intensive degradation of structural units to convert complex sugars into simple sugars like glucose. On the other hand, the maximal sugar were comparable to other similar biomasses. Kuglarz et al. (2018), reported that rapeseed contains 27.80 g/L of total sugars (glucose, xylose, arabinose) and 23.30 g/L reducing sugar (glucose) after 1% H_2SO_4 acid pretreatment and enzymatic hydrolysis. The results of our work from PPR and CPRS are higher due to higher acid concentration, which were found significant on statistical analysis (see Table 1 and 2) Consequently, results were similar to the works of (Mikulski and Kłosowski, 2018; Sahoo et al., 2018) whereas the optimum sugars was also achieved at 2% concentration of H_2SO_4. Meanwhile, others also reported promising concentration of sugars using sequential pretreatment. Raghavi et al. (2016) treated the sugarcane trash using sequential ferric chloride and sodium hydroxide and produced 0.80 g/g of reducing sugar. Additionally, coffee spent waste from two-step pretreatment and hydrolysis has achieved 0.35 g/g of fermentable sugars. Overall, this implies that sequential pretreatment applied on coconut pulp residue is capable of producing higher sugar concentration needed for fermentation, which was also found significant based on statistical analysis.

4. Conclusion

The coconut pulp residue was treated sequentially by hydrothermal and acid pretreatment and was optimized using response surface methodology. The results revealed the maximal total and reducing sugar could be attained from 40 minutes pre-hydrothermal treatment time and 2% H_2SO_4 for

PPR and 20 minutes hydrothermal treatment time and 2% H_2SO_4 for CPRS. In this condition, the sugar concentration were 252.46 g/L (0.51 g/g) and 153.97 g/L (0.31 g/g) for PPR while in CPRS 253.71 g/L (0.51 g/g) and 207.32 g/L (0.42 g/g), respectively. To the best of our knowledge, this is the first report on sequential pretreatment of coconut pulp residues using hydrothermal and acid pretreatment which was highly effective in producing significant amount of sugar which can serve good source for bioethanol production.

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