Effect of sodium hydroxide pretreatment on released sugar yields from pomelo peels for biofuel production

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Abstract. Most of the agricultural wastes in developing countries are disposed of by on-site combustion leading to unmanaged environmental pollutions. Conversion of agricultural wastes to value-added products, such as bioethanol and biogas, is a promising method to reduce agro-waste after harvesting seasons. In this study, *Citrus maxima* peels (Pomelo peels) was selected to be converted to reducing sugars, which could be a raw material to produce other value-added products. To promote enzymatic hydrolysis reactions, pomelo peels were pretreated with sodium hydroxide by variations of three pretreatment parameters, including temperature (50-100 ºC), time (0.5-6 h), and concentration of NaOH (0.5-3.0 M). Box-Behnken design (BBD) was applied in Response Surface Methodology (RSM) to determine the optimized pretreatment conditions and to find the relationship between pretreatment factors and reducing sugar yields. The predicted optimal pretreatment condition was determined to be at 94.28 ºC, 4.5h, 2.17M with reducing sugar yield of 98.9 mg/g of dried pomelo peels. The results clearly showed that reducing sugar yields obtained from pretreated pomelo peels were 1.87 folds higher than untreated biomass (52.81 mg/g of pomelo peels). Therefore, this study demonstrated the potential of pomelo peels to be used as an alternative raw material for value-added products rather than being a landfill or causal agent of pollution.

Keywords: Sodium hydroxide, Pretreatment, Enzymatic saccharification, Biorefinery, Biofuels

1 INTRODUCTION

Energy consumption has increased in the past decade across the globe. An increase in population rate along with industrial development has led to more energy consumption than in the past years. Energy production using non-renewable sources is insufficient to meet the rising energy demand. This has led to an increase in the research on renewable energy produced from hydropower, solar energy, wind power, and biomass. Many researchers have shifted their focus to biomass for energy production since it is widely available [1].

Thailand is a country rich in agricultural resources and produces a large amount of agricultural waste, such as straw, rice husk, coconut shell, sawdust, leaves, bark, fruit husk, corncob, and bagasse, etc. According to the data in 2013, Thailand produced 62 million tons of biomass per year, which had the potential to generate 13,348 thousand tons of crude oil [1]. Statistic data show the possibility of utilizing agricultural waste biomass for energy production. For instance, fruit peels from canned fruit factories could be utilized to produce biogas or can be processed into fertilizers or animal feeds. Currently, most of the residual biomass after processing in the industry is either landfilled or incinerated, which causes fine particle release with serious health and environmental concerns. Instead of this, residual biomass could be used to generate renewable energy for local use. This not only provides health benefits for people but also reduces environmental pollution [2].

Biomass materials, such as agricultural processed fruit peels, are mainly composed of lignocellulose materials. Lignocellulose is a complex material with a rigid structure. Lignocellulose is composed of 3 mains components, namely cellulose, hemicellulose, and lignin [3,4]. Cellulose is the main component, which is a homopolymer of glucose connected with β-1, 4 linkages to form a long polymeric chain. Hemicellulose is a heteropolymer composed of repeated units of pentoses and hexoses. Lignin is an aromatic polymer that provides the rigidity for biomass. Each plant material has a varying amount of these components depending on its species, growth stage, and climate [5-7]. These components are arranged in a specific manner in the biomass to make it rigid and recalcitrant. The rigid

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nature of biomass greatly hinders the activity of enzymes or microorganisms to degrade biomass to produce energy. Therfore, a preliminary process to facilitate the disintegration of biomass, before its utilization is necessary.

In general, there are four important steps in bioenergy production, namely the pretreatment process, followed by hydrolysis, fermentation, and bioenergy extraction [8,9]. The pretreatment process is conducted to make biomass become vulnerable to enzyme activity. Pretreatment can be done in several ways, such as physical pretreatment)milling or grinding([10-11], chemical pretreatment)alkali, dilute acid, oxidizing agents and organic solvents, ionic liquid([12-20], biological pretreatment [21-24] and, physicochemical pretreatment [10,11]. Each method has its advantages and disadvantages. However, these pretreatment methods can increase porosity and surface area to the biomass structure [25]. This provides more surface area for the enzyme to react and increases the hydrolysis efficiency by increasing sugar yield at a reduced cost of enzyme for cellulose digestion [26]. Chemical pretreatment using acid helps in the digestion of hemicellulose and lignin to produce high sugar yield and generate inhibitors or furfural [7]. Whereas, alkaline pretreatment, using solutions such as sodium hydroxide, calcium hydroxide, and ammonia helps in the removal of lignin and some amount of hemicellulose. Pretreatment using these chemicals can cause degradation of ester and glycosidic side chains causing the cellulose to swell. This also helps in decreasing crystallinity and polymerization thereby increasing internal surface area by destroying the lignin structures [27]. Pretreatment is followed by intensive washing to remove any trapped impurities in the solids after pretreatment [28]. This can enhance the efficiency of the enzyme in the hydrolysis process [29] and thereby increases the yield of required products, such as bioethanol [26].

In this research, pomelo peels was targeted in our investigation in the production of fermentable sugars, which could subsequently be converted to value-added products or biofuels. To improve the efficiency of enzymatic saccharification of pomelo peels, the pretreatment process with sodium hydroxide was optimized based on Response Surface Methodology (RSM) with Box-Behnken design (BBD). The maximum amount of sugars produced from this work could be used as critical data for process design in the biorefining process of lignocellulosic biomass.

2 Material and methods.

2.1. Raw materials

Pomelo peels were obtained from a local market in Nonthaburi province in Thailand. Pomelo peels were dried at 60 °C for 12 h. In hot air oven to remove moisture from them. After drying, it was cut by using house blender thoroughly and particle sizes were screened through a 20 mesh-sized aluminum sieve to obtain sample consistency. The biomass was stored in a sealed plastic bag at room temperature until further use.

CelluClast 1.5 L from *Trichoderma reesei* was purchased from Sigma-Aldrich (aqueous solution 700 units / g), β-glucosidase was obtained from Megazyme, Sodium hydroxide (NaOH), and other analytical reagents were purchased from Alex Fincohem (Univar).

2.2 Alkaline pretreatment

A pretreatment was carried out by immersing 5 g of pomelo peels in 50 ml of NaOH solution at a solids loading of 10%. A treatment was performed in hot air-oven using varied NaOH concentration (0.5 to 3 M), temperature (70 to 100°C) and time (1 to 24 hours) to preliminarily find the boundary of testing factors. After pretreatment, the solid residues were removed from the liquid fraction by filtration with Whatman No. 1 filter paper. The solid residues were further washed with distilled water until neutral pH. The solids were dried in a hot-air oven at 60 °C for 8 hours. Pretreated samples were stored in a desiccator before proceeding to enzymatic saccharification.

2.3 RSM Experiment design

To determine the optimal pretreatment condition for producing maximum sugar yield (Y), BBD of RSM was selected. RSM experiments were designed with 3 pretreatment factors, namely pretreatment temperature (X_1), Pretreatment time (X_2), and NaOH Concentration (X_3). Pretreatment was carried out at varied NaOH concentrations of 0.5-3 M, the temperature of 50-100 °C, and time of 0.5-6 h. Each of these factors was set at three different levels -1, 0, and +1 (Table 1). A total of 17 runs were performed by varying conditions of pretreatment factors as in Table 2. The pretreated biomass was further hydrolyzed by the enzyme to determine the reducing sugar yield. The RSM experiments were design and experimental data were analyzed by using Design Expert version 7.0.0 software.

Table 1. Independent variable factors in pretreatment condition of pomelo peels on RSM.

Independent variable factors	Coded symbols	Levels		
		-1	0	+1
Temperature (°C)	X_1	50	75	100
Time (h)	X_2	0.50	3.25	6
Concentration (%)	X_3	0.50	1.75	3

2.4 Enzymatic hydrolysis

Untreated and pretreated pomelo peels were enzymatic hydrolyzed by commercial cellulase. The hydrolysis reaction was carried out using 0.1 g biomass in 4 ml of hydrolysis buffer (containing 50 mM citrate buffer, 40 µl of 2M sodium azide, 35µl of CelluClast 1.5 L, and 10µl of β-glucosidase enzyme). The reaction mixture was incubated at 50 °C, 150 rpm for 72 hours. The reaction was stopped by incubating the reaction mixture at 100 °C for 10 minutes. Biomass hydrolysates were harvested by

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centrifugation at 10,000 g for 5 minutes. The supernatant was used to analyze for reducing sugar concentration after enzymatic hydrolysis.

Table 2. RSM experimental design for NaOH pretreatment of pomelo peels

Run	Pretreatment condition			Reducing sugar (g/100g biomass)
	X_1: Temperature (°C)	X_2: Time (h)	X_3: Concentration (M)	
1	100	3.25	0.5	56.80
2	50	6	1.75	43.44
3	75	0.5	0.5	37.85
4	100	3.25	3	97.76
5	50	3.25	3	47.07
6	75	0.5	3	41.69
7	75	3.25	1.75	75.04
8	50	0.5	1.75	40.44
9	100	0.5	1.75	45.05
10	75	3.25	1.75	79.68
11	75	6	3	98.15
12	100	6	1.75	91.31
13	75	6	0.5	57.96
14	75	3.25	1.75	78.73
15	50	3.25	0.5	29.94
16	75	3.25	1.75	80.27
17	75	3.25	1.75	80.84

2.5 Analysis of reducing sugar

The reducing sugar concentration was analyzed by the dinitrosalicylic acid (DNS) method [30]. Briefly, 50 mL of supernatant from enzymatic hydrolysis reaction was mixed with 150 mL of DNS solution. The mixture was incubated at 95°C in a water bath for 5 minutes. The mixture was incubated on ice for 5 minutes. After incubation, 1 mL of distilled water was added to the mixture and it was mixed well with a vortex shaker. The reducing sugar concentration was measured using a UV / VIS spectrophotometer at 540 nm. The concentration of reducing sugar was calculated based on a glucose standard curve (Figure 1). The effects of various pretreatment conditions on reducing sugar concentration were statistically analyzed by ANOVA.

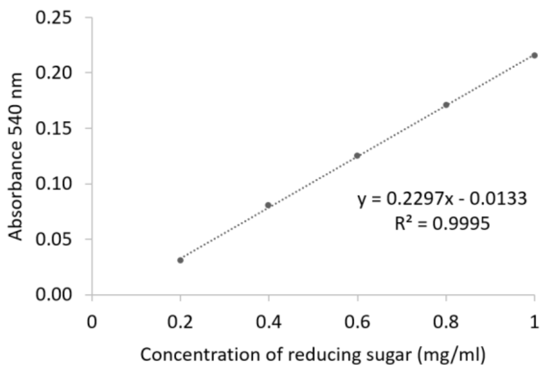


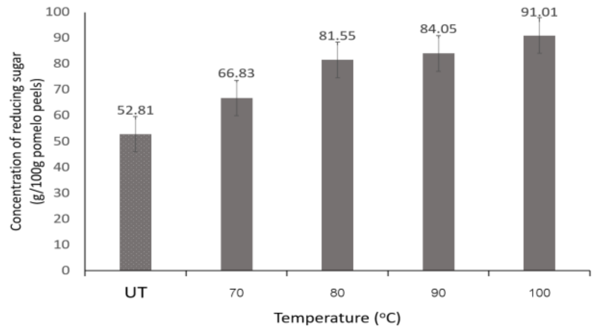
Fig. 1. Standard glucose curve for DNS method.

3 Result and discussion

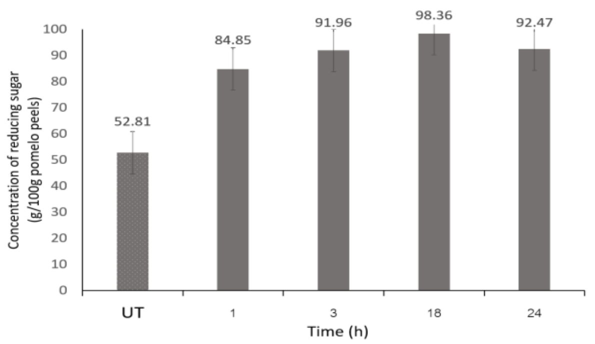
3.1. Alkaline pretreatment of Pomelo peels

An alkaline pretreatment of pomelo peels was carried out to understand the range of pretreatment factors to be used in the RSM study for optimization of pretreatment conditions based on the sugar yield. Initially, pomelo peels was pretreated varying pretreatment temperature from 70 to 100 ℃ while keeping NaOH concentration and pretreatment time constant at 2 M and 1 hour respectively. The results clearly show that the yield of reducing sugar has increased from 52.81 g to 91.01 g/ 100 g pomelo peels when the pretreatment temperature was increased (Figure 2a).

(a)



(b)



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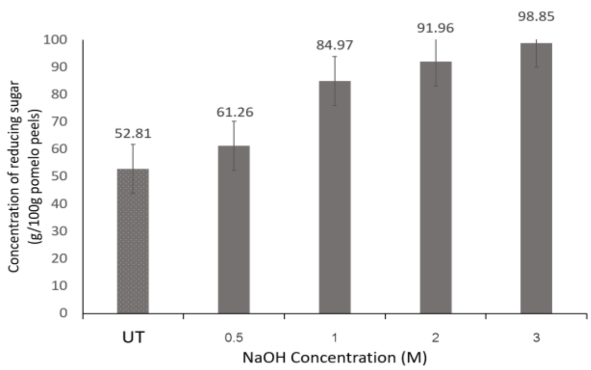

Fig. 2. Effect of pretreatment temperature, time and NaOH concentration on the enzymatic hydrolysis of Pomelo peels. (A) The pretreatment was using 2M of NaOH for 1 hour, (B) The pretreatment was using 2M of NaOH at 80°C and (C) The pretreatment were conducted at 80°C for 3 hours.

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In comparison with the untreated biomass, there was a 1.72-fold increase in the sugar yield when pretreated at 100℃. Similarly, when the pretreatment was carried out by varying the NaOH concentration from 0.5 to 3 M, at 80 ℃ for 3 hours, the result showed that the reducing sugar yield increased with the increasing NaOH concentration (Figure 2b). A maximum sugar yield of 99.52 g/ 100 g of biomass was obtained at 3 M NaOH concentration, which is 1.88 times more than untreated pomelo peels. Unlike this, when the pretreatment was performed by varying time from 1-24 hours, with 2 M NaOH at 80 ℃, the reducing sugar yield increased up to a certain pretreatment time, after that the yield was reduced (Figure 2c). The reducing sugar yield increased from 52.81 g/100 g biomass to 98.36 g/ 100 g pomelo peels by 18 hours of pretreatment. However, when the pretreatment time was further extended to 24 hours, the reducing sugar yield decreased to 92.47g / 100 g pomelo peels. This could be possibly due to the destruction of the internal structure of the biomass caused by the long pretreatment time. Also, the sugar yield did not show much variation when the pretreatment time was varied from 3 hours (91.96 g/ 100 g biomass) to 18 hours (98.36 g/ 100 g biomass). Hence, to reduce the pretreatment cost and time, the time range for pretreatment at the RSM study was chosen between 0.5 and 6 hours.

3.2. RSM design and experimental testing

The RSM is considered an efficient method to design and conduct experiments to achieve the optimal state in the production process. RSM evaluates the effects of individual independent variables and their interactions with RSM-dependent variables and also helps to reduce the number of experimental trials. It is widely used to reduce the time and cost of the experiment. In addition to this, RSM can also predict results and can create surface models under different conditions of the independent variables. Several studies have applied RSM to improve the efficacy of their studies [31-34].

In this study, RSM created the BBD matrix with 3 levels of testing: low (-1), mid (0), and high (+1) for three factors used for the pretreatment (pretreatment time, pretreatment temperature, and NaOH concentration). A total of 17 runs were conducted. The statistical analysis was performed using Design-Expert software (ver. 7.0.0) throughout this research. The experimental data were used to find the experimental coefficient (β) using the least-squares method. In addition to this, the second-order model was generated from the experimental data. The optimum pretreatment conditions were determined based on the highest yield of reducing sugar from the multiple regression analysis of the second-order model. The correlation of the second-order model was assessed by determination of the coefficient (R^2), the significance of the test condition in ANOVA, and the lack of fit, which is generally considered significant when the R^2 value is greater than 0.9 [31-34]. In this paper, R^2 value of model was 0.9396, which indicated the lower variance during the experiment and reliability of the model. ANOVA was further conducted to determine the importance of the second-order model (Table 3).

Table 3. ANOVA analysis of the second-order model obtained from RSM experiments.

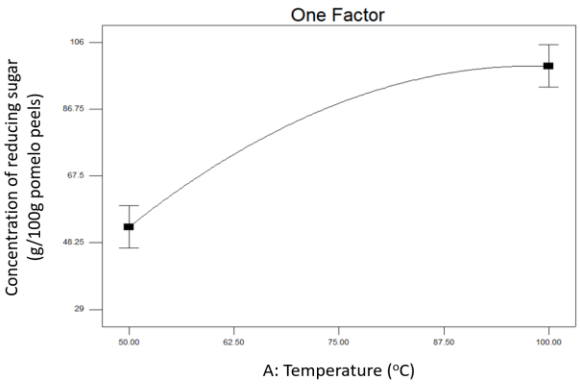
Source	Sum of Squares	df	Mean Squares	F Value	P-value Prob > F
Model	8018.51	9	890.95	28.65	< 0.0001
A-Time	2113.48	1	2113.48	67.96	< 0.0001
B-Time	1979.15	1	1979.15	63.64	< 0.0001
C-Conc	1303.56	1	1303.56	41.92	0.0003
A^2	651.18	1	651.18	20.94	0.0026
B^2	548.74	1	548.74	17.65	0.0040
C^2	310.22	1	310.22	9.98	0.0160
Residual	217.68	7	31.10		
Lack of Fit	196.5	3	65.50	12.37	0.0172
Pure Error	21.18	4	5.29		
Cor Total	8236.19	16			

The ANOVA analysis of the model was found to have an F value of 28.65 with statistical significance ($p < 0.05$) and a 0.01% chance of the model F-value noise. Moreover, pretreatment temperature, pretreatment time, NaOH concentration were also significant pretreatment factors according to ANOVA analysis. The lack of fit is 12.37 which was also considered significant and has a 1.72% chance of noise. However, according to the data we have found that there was a small chance that the model will not fit and distributes anomalies. According to the ANOVA analysis above, it was estimated that all three parameters : pretreatment temperature, pretreatment time, NaOH Concentration affected the reducing sugar production after pretreatment of pomelo peels. Other studies have also used such parameters as L. Kim et al [35] performed alkaline pretreatment of Rice straw by using NaOH under various conditions of time, temperature and NaOH concentration to find a correlation between reducing sugar content and pretreatment parameters, In addition, P. Amnuaycheewa *et. al*. [36] performed acid pretreatment of Napier grass by using HCL under various conditions of time, temperature and HCL concentration to reported a similar correlation between reducing sugar contents and pretreatment parameters, so it was found that these parameters were related to reducing sugar content.

3.3 Effect of pretreatment parameters on the enzymatic saccharification on mathematical models

The impact of each factor, pretreatment temperature, pretreatment time, NaOH Concentration on the response of the curve was analyzed based on the concentration of reducing sugar (Figure 3). The model also analyzed the effect of the interaction between independent variables (pretreatment factors) in the form of a three-dimensional contour plot. The data showed that the yield of reducing sugar increased as the pretreatment temperature (Figure 4a), pretreatment time (Figure 4b), and NaOH concentration increased (Figure 4c). This implied that all three parameters affected the sugar yield. The more severe pretreatment conditions could lead to the swelling in the cellulose structure, leading to an increase in the surface area. This provided more accessibility for the enzyme to cellulose leading to enhanced sugar production.

(a)



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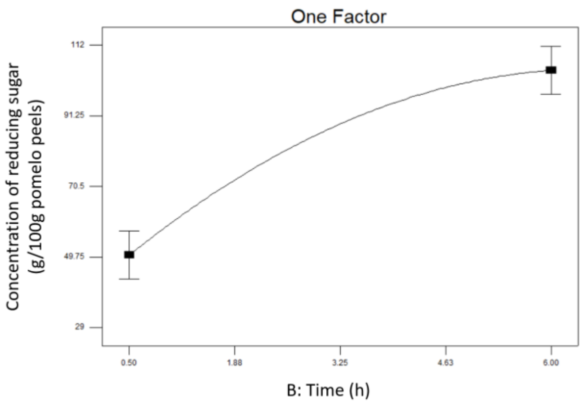

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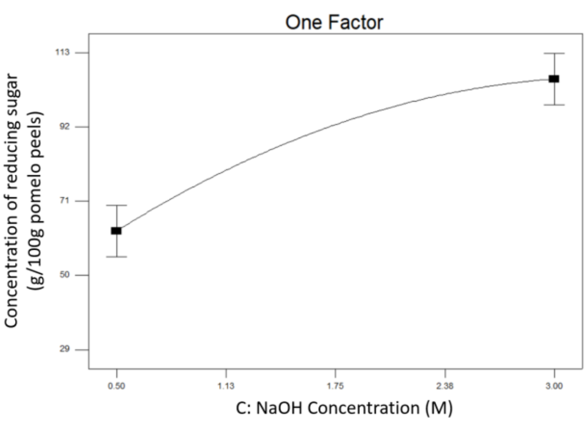
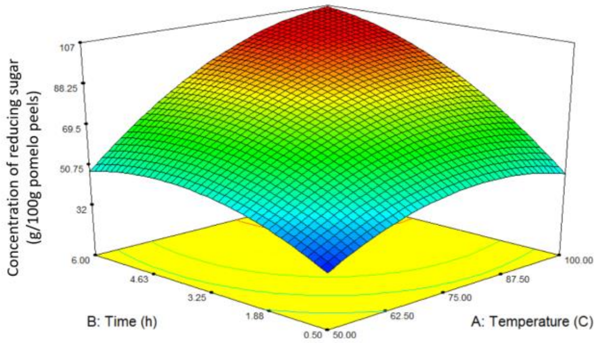


Fig. 3. Relationship between each pretreatment factor, (A) pretreatment temperature (°C), (B) pretreatment time (h), (C) NaOH concentration (M) and Y-axis is concentration of reducing sugar (g/100g pomelo peels)

(a)



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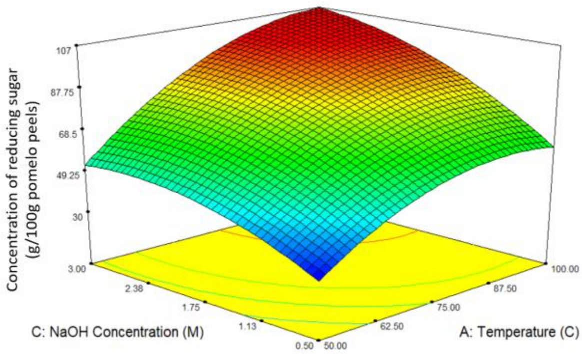

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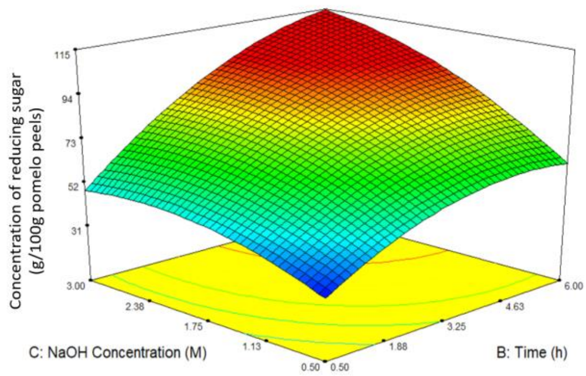

Fig. 4. Response surface plot represents the effects of pretreatment factors on reducing sugar yield (g/100g pomelo peels). (A) pretreatment temperature vs. pretreatment time, (B) pretreatment temperature vs NaOH concentration and (C) pretreatment time vs NaOH concentration

ANOVA analysis could simulate a model and predict optimal parameters for the pretreatment. The mathematical model suggested pretreatment using 2.17 M NaOH at 94.28 °C, for 4.5 hours as the optimal pretreatment conditions for pretreating Pomelo peels to yield maximum sugar yield. (Table 4). The model also predicted that the optimal pretreatment condition could yield 98.9 mg reducing sugar per gram of pomelo peels. To confirm the reliability of this model, pretreatment was conducted again at the predicted optimal pretreatment conditions. It could yield 99.1 mg reducing sugar per gram pomelo peels with only 0.2% error from the predicted value. When comparing the yield of reducing sugar, it could be deduced that the model from ANOVA prediction was highly accurate

Table 4. Mathematical models and optimal pretreatment condition obtained from RSM experiment.

Mathematical Model		
Reducing sugar = -72.59982 + 2.78992 x Temp - 0.89290 x Time + 6.54922 x Conc + 0.15731 * Temp * Time + 0.19064 * Temp * Conc + 2.64364 x Time x Conc - 0.019898 x Temp ^{2} - 1.50955 * Time^2 - 5.49344 x Conc^2		
Optimal pretreatment parameter		
Temperature(°C)	**Time (h)**	**NaOH Concentration(M)**
94.28	4.5	2.17
Predicted Concentration of sugar (g/100g pomelo peels)	Experimental Concentration of sugar (g/100g pomelo peels)	Difference (%)
98.90	99.10	0.2

4 Conclusion

The study was conducted to achieve maximum yield of reducing sugar from optimally pretreated pomelo peels and these sugars could be further converted to products such as biogas, bioethanol, etc. In this research, the optimization of pretreatment conditions was carried out using the RSM model. The preliminary alkaline pretreatment studies could help us in determining the range of pretreatment factors to be used in the RSM model. BBD of RSM was applied to design the experiments with various pretreatment conditions by varying NaOH concentration, pretreatment temperature, and pretreatment time. The BBD design along with the ANOVA analysis predicted NaOH concentration of 2.17M, pretreatment temperature of 94.28 °C, and pretreatment time of 4.5 hours as optimal pretreatment condition, which could yield of reducing sugars of 98.9 mg / g of pomelo peels. This implied a 1.87 fold increase in sugar yield obtained from pretreated biomass compared to untreated biomass. This study demonstrated the potential of pomelo peels to be used as feedstock in the biorefining process. Also, it provided an alternative way to reduce agriculture waste and air pollution.

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