Oxalic Acid Pretreatment on Enhancement of Enzymatic Saccharification from Napier Grass for Biofuel Production

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Abstract. Thailand as an agricultural country faces significant challenges in managing the abundant biomass waste generated from agricultural activities. Conventional disposal methods such as incineration contribute to pollution and limited availability of landfill space. To mitigate these issues valorization of this biomass waste has been a solution. This study focuses on the utilization of Napier grass as a renewable energy source. In this experiment, the Napier grass samples were pretreated using oxalic acid with temperature variations (50 – 100 °C), time (30 - 180 min), and oxalic acid concentration (2 - 10%w/v) to determine the limit of these three factors for optimization studies. The utilization of Box-Behnken Design (BBD) within Response Surface Methodology (RSM) enabled the determination of optimal pretreatment conditions and the exploration of the correlation between pretreatment factors and reducing sugar content. The model predicted pretreatment with an oxalic acid concentration of 6% w/v, pretreated at 100 °C for 105 min as the optimal pretreatment condition to produce a maximum reducing sugar concentration of 10.65 mg/ml. Therefore, the sample was pretreated at optimum conditions and the results revealed the amount of reducing sugar obtained was 10.67 mg/ml, which differed from the predicted value with an error of 0.22%. Thus, this study provides insight for future researchers on the optimum condition that can be applied for pretreating biomass with oxalic acid to maximize the sugar yield.

Keyword. Bioethanol, Biorefinery, Enzymatic hydrolysis, Organic acid, Oxalic acid, Pretreatment

1 Introduction

The global population explosion has led to a surge in energy demand across the world. This surge in energy demand is posing a significant challenge to global energy security. The energy production from fossil fuels is hence limited and has facilitated the research on alternative sources for energy production from renewable resources [1]. Thailand is a country with agriculture in all regions, resulting in a large amount of biomass waste from agriculture. These waste materials are mostly used as household heat sources by combustion. Some of these waste materials are either incinerated or dumped in landfills [2]. Apart from its agricultural sector, Thailand is also recognized as an industrial nation, particularly in the food production industry [3]. Consequently, there is a generation of waste materials following the completion of mass production processes. Examples of such waste materials include fruit pulp from juice factories, vegetable waste from food or snack manufacturing facilities, and various byproducts from sugar factories, such as sugarcane residues. These industrial waste materials pose environmental challenges and require proper management. These agro-wastes are often sold cheaply to farmers to produce animal feed [4]. Therefore, biomass energy is considered a renewable energy alternative to fossil fuels to increase the energy supply to meet the demand. Since fossils are an exhaustive source of energy, the utilization of biomass helps reduce global warming caused by the combustion of fossil fuels.

Napier grass is classified as lignocellulose biomass material. Its main components are cellulose, classified as a polysaccharide carbohydrate with glucose molecules connected in a long chain; hemicellulose, a heterologous polymer composed of pentose and hexose subunits, and lignin, an aromatic polymer [5]. These components are interconnected in a complex manner providing recalcitrance to the plant cell. The amount of these components varies in each plant [6]. Previous studies have already identified cellulose and hemicellulose to have the potential to be used as feedstock for energy production from biomass to produce bioethanol from it [7].

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The complexity of biomass structure has resulted in the application of advanced technology such as a pretreatment process to break down its structure to a simpler form. This step will help in breaking down the polymers into monomers and oligomers that can be taken to the downstream processing steps and finally converted to bioethanol [8]. Among various types of pretreatment methods, including physical, chemical, physicochemical, and biological approaches, chemical pretreatment is extensively employed for biomass utilization. [9,10]. Among the various types of chemical pretreatment, acid pretreatment, and alkaline pretreatment are commonly used techniques [8, 11, 12]. Acid pretreatment can be done with both organic and inorganic acids, however, the use of inorganic acid generates more inhibitors and can cause corrosion to the equipment than the organic acids [13] and it has the benefit of environmental protection [14]. The selection of the pretreatment technique will depend on the purpose of biomass pretreatment.

Oxalic acid is an organic acid that has been applied for the pretreatment of various types of biomass. Pretreatment of olive tree biomass was performed using oxalic acid for production of biohydrogen based on Response Surface Methodology (RSM). A dark fermentation process was carried out using an OTB hydrolysate to maximize the yield of reducing sugar. The independent variables considered in the experiment were time (30–90 min), temperature (100–140 ºC), and acid concentration (5–10% w/w for oxalic acid). Under the optimal pretreatment conditions, the maximum reducing sugar obtained was 28 g/L. Furthermore, the highest hydrogen yield achieved through oxalic acid pretreatment was 0.83 mol H2/mol reducing sugar. [15]. Waste lignocellulose medium after cauliflower mushroom (*Sparassis crispa*) cultivation was pretreated by using diluted oxalic acid. The pretreatment was conducted by using the total solids/liquid ratio of 1:4 by weight and the glucose yield was obtained at 44.662 g/l [16]. Corn cob pellets were pretreated with 3% oxalic acid and a solid loading ratio of 1:6. The pretreatment was conducted in a vacuum reactor at 168 ºC for 26 min. The main products obtained after saccharification were 2.74 g/L of glucose, 32.55 g/L of xylose, and 1.71 g/L of arabinose. However, just a small fraction of lignin was removed by oxalic acid pretreatment [17]. Yellow poplar (*Liriodendron tulipifera*) stems were pretreated with 3.7% oxalic acid at a loading ratio of 1:4 and 160 ºC, and total sugars were obtained at 33.7 g/L [18]. These studies demonstrated the potential of oxalic acid for the pretreatment of lignocellulose.

In this study, Napier grass will be pretreated with organic acid as it is less toxic and causes no corrosion to the equipment. Moreover, it also generates less inhibitory compounds that can interfere with the enzyme in the hydrolysis process causing a decrease in the yields of the reducing sugar that can be converted to bioethanol. The organic acid used in this research is oxalic acid. Response surface methodology (RSM) was employed to optimize the pretreatment conditions and enhance the sugar yield from the biomass. The concentration of reducing sugar was determined using the 3,5-dinitro salicylic acid (DNS) assay.

2 Material and Methods

2.1. Preparation of Raw Material

The Napier grass used in this study was sourced from Kanchanaburi province in Thailand. To remove moisture, the biomass was dried at 60 ºC in a hot air oven. Subsequently, the biomass underwent size reduction by using a household blender and was sieved through a 20 mesh-sized aluminum sieve to achieve a uniform particle size. Finally, the sample was stored in a sealed plastic container.

2.2 Acid Pretreatment

Napier grass was pretreated with 2 - 10% w/v of oxalic acid concentration at 50 - 100 ºC for 30 - 180 min with 10% biomass loading to determine the limits of the test factors. After the pretreatment, the pretreated slurry was centrifuged to separate the solid fraction from the slurry. The pretreated solids were washed with distilled water and neutralized with 1 M of sodium hydroxide. Further, the solids were separated by vacuum filtration with Whatman filter paper No. 1 and dried at 60 ºC for 12 h. The Samples were stored in a desiccant jar until further use.

2.3 RSM Experimental Design

The response surface methodology was chosen in the experiment to identify the optimal conditions for pretreatment based on three factors: time, temperature, and oxalic acid concentration. Each factor was set to three different levels, consisting of low, medium, and high levels, denoted by the symbols -1, 0, and +1 respectively (Table 1). All 17 experiments of pretreatment (Table 2) were derived from RSM design and will be hydrolyzed to determine the reducing sugar after pretreated based on the ANOVA analysis (p–value < 0.05) to find the correlation of pretreatment parameters. Based on the coefficient of determination (R^2), the significance of the condition test in ANOVA, and the lack of fit, the multiple regression analysis will enable the prediction of the optimal condition that yields the highest amount of reducing sugar. Additionally, a second-order model was generated for this purpose.

2.4 Enzymatic Hydrolysis

The pretreated and untreated Napier grass samples were enzymatically hydrolyzed using a commercial enzyme, CTec2. The hydrolysis reaction was performed using 1 g of biomass and a hydrolysis buffer solution consisting of 40 ml of 50 mM citrate buffer, 400 µl of 2M sodium azide, and 200 µl of CTec2. The reaction was conducted at 50 ºC and 150 rpm for a duration of 72 hours [19,20].

2.5 Analysis of Reducing Sugar

The concentrations of reducing sugars in the enzymatic hydrolysate were analyzed by the DNS assay [21]. Briefly, a total of 50 µl of hydrolyzed supernatant was combined with 150 µl of DNS solution. The resulting mixture was incubated at 95 ºC in a water bath for 5 min. To halt the reaction, the mixture was promptly cooled on ice for 5 min. Subsequently, 1 mL of distilled water was added to the mixture. The concentration of reducing sugars was determined by measuring the absorbance at 540 nm using a UV/vis spectrophotometer. The concentration was calculated using a glucose standard curve (Figure 1).

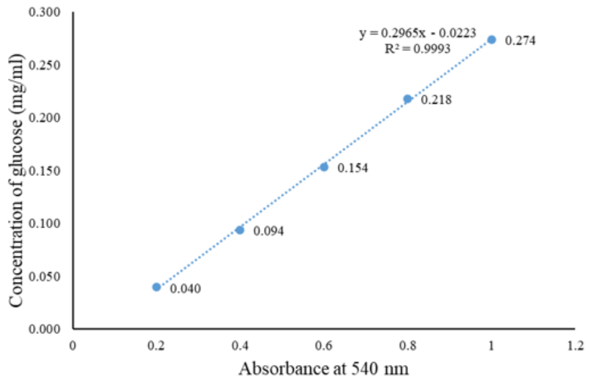


Fig. 1. Standard curve of glucose**.**

2.6 FTIR Analysis

Fourier Transform Infrared (FTIR) spectroscopy was carried out on both untreated and pretreated Napier grass samples to determine the chemical structures and chemical bonding arrangements using BRUKER, INVENIO S with a resolution of 4 cm^{-1} from 400 cm^{-1} to 4000 cm^{-1}[22].

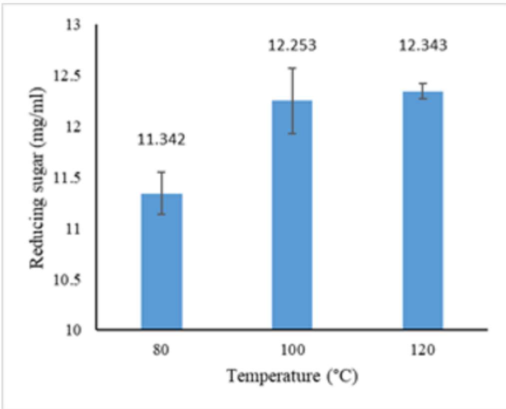
3 Results and Discussion

3.1 Acid Pretreatment of Napier Grass

In order to assess the range of the test factors used in the RSM investigation, a comparative analysis was conducted, encompassing three variables: pretreatment temperature (ranging from 50 to 100 ºC), pretreatment time (ranging from 30 to 180 min), and oxalic acid concentration (ranging from 2 to 6% w/v). This experiment was carried out to determine the boundaries of the variables used in the RSM design. The boundaries of each variable were selected based on the maximum reducing sugar concentration. To determine the temperature boundary, Napier grass samples underwent pretreatment with 6% (w/v) oxalic acid for 105 min, with the temperature ranging from 50 ºC to 100 ºC (Figure 2A). The experiment demonstrated that pretreatment at 100 ºC yielded the highest amount of reducing sugar, producing 12.556 mg/ml. Increasing the temperature further to 120 ºC did not have a significant impact on the concentration of reduced sugar. Therefore, the upper limit for the pretreatment temperature was established as 100°C.

Determination of boundary for the time was carried out by pretreating Napier grass samples with oxalic acid at 6%w/v at 100 ºC, for durations varying from 30 to 180 min (Figure 2B.). The result shows an increase in the sugar concentration when the duration was extended from 30 min to 105 min. In Figure 2B, the error bands overlap between the pretreatment for 105 and 108 min, indicating that pretreatment with such different duration is not a factor that affects the concentration of reducing sugars. However, further extending the time to 180 min led to a reduction in the sugar concentration, suggesting limiting the pretreatment time to 105 min. The boundary limit for oxalic acid concentration was determined by pretreating the biomass with varying concentrations of oxalic acid from 2-10% w/v at 100 ºC for 105 min (Figure 2C). Increase in the oxalic acid concentration initially showed an increase in the sugar concentration. A further increase to 10%w/v showed a decrease in the sugar concentration.

A



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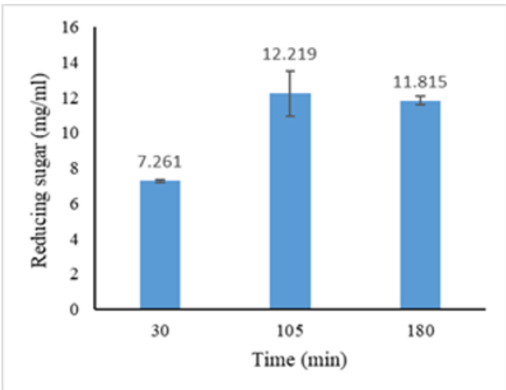

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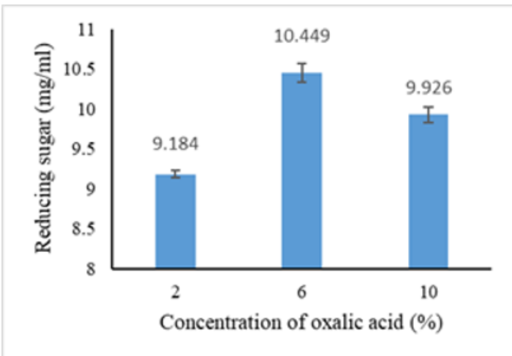


Fig. 2. Effect of pretreatment temperature(A), time(B), and 2 - 6% of Oxalic concentration(C) on the enzymatic hydrolysis of Napier grass. A; the pretreatment was using 6% of Oxalic acid for 105 min, B; the pretreatment was using 6% of Oxalic acid and 100 ºC, C; the pretreatment was conducted at 100 ºC for 105 min.

Therefore, for this pretreatment, the boundary of factors used in the RSM design was selected as follows: temperature ranging from 50 to 100 ºC, time varying from 60 to 105 min, and oxalic acid concentration between 2 and 6% (w/v). These 3 factors were further used to design experiments with RSM (Table 1 and Table 2) by using the Design - expert software (version 7.0.0).

Table 1. Pretreatment factors obtained from RSM with BB design.

Variable factors	Coded symbols	Levels		
		-1	0	+1
Temperature (c)	X_1	50	75	100
Time (min)	X_2	60	82.5	105
Oxalic acid concentration (%)	X_3	2	4	6

Table 2. 17 Experimental runs from RSM design.

Run	Pretreatment condition			Reducing Sugar concentration (mg/ml)
	Temp (°C)	Time (min)	Conc (%,w/v)	
1	50	82.50	6	5.98
2	75	60	2	8.07
3	75	82.50	4	7.23
4	50	60	4	7.13
5	50	82.50	2	7.56
6	75	82.50	4	7.06
7	75	82.50	4	7.09
8	75	105	2	7.70
9	100	82.50	6	8.07
10	100	105	4	11.24
11	100	60	4	7.70
12	75	82.50	4	7.16
13	75	82.50	4	7.19
14	75	60	6	7.94
15	100	82.50	2	7.26
16	50	105	4	5.98
17	75	105	6	7.63

3.2 RSM Design and Experimental Testing

Response Surface Methodology (RSM) is a practical application of mathematical and statistical techniques that prove valuable in the modeling and analysis of problems. The response of interest depends on many variables and the objective is to find the best value of the response [23]. Therefore, RSM is an effective technique for complex processes. It reduces the cost and time spent on experiments, which makes it easier to manage and explain the results when compared with other methods [24, 25, 26]. Among the various designs in RSM, Box-Behnken Design was chosen to design the experiment. The Box-Behnken Design is a three-level design for the rebound surface fit. This design is built on combining the 2^K factorial design with an incomplete block design [27,28]. Analysis of variance (ANOVA) is a method to test the difference between the mean of samples from 3 or more groups. This will analyze the ratio of the variance between groups and within the group. The least - Squared method was used to find the experimental coefficient (β) by generating the second-order model from the experimental data. The optimal condition for pretreatment was determined by analyzing the highest yield of reducing sugar concentrations obtained from the multiple regression analysis of the second-order model. [29].

Table 3. ANOVA analysis for the mathematical model.

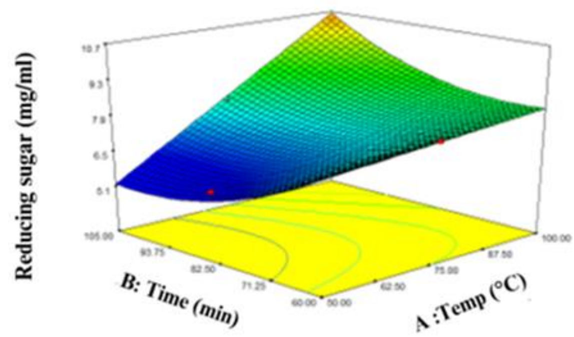
Source	Sum of Squares	df	Mean Squares	F Value	p-value Prob>F
Model	16.54	4	4.14	12.93	0.0003
A-Temp	7.26	1	7.26	22.70	0.0005
AB	5.50	1	5.50	17.20	0.0014
AC	1.43	1	1.43	4.47	0.0562
B^2	2.36	1	2.36	7.37	0.0188
Residual	3.84	12	0.32		
Lack of Fit	3.82	8	0.48	96.79	0.0003
Pure Error	0.020	4	4.930E-0.03		
Cor Total	20.38	16			

The ANOVA analysis of the model was found to have an F value of 12.93 with a p - value <0.05 and the chance of the model F - value noise is 0.03%. The lack of fit is 96.79 with a 0.03% chance of noise. The ANOVA analysis suggests the significance of the model (p-value = 0.0003). From the data shown in Table 3, The relationship between AC (A: temperature and C: oxalic acid concentration) had a p-value of 0.056, which was over 0.05, indicating that this relationship was not statistically significant. Therefore, it was not used to generate a mathematical model.

3.3 Influence of Pretreatment Parameters on the Enzymatic Hydrolysis of Mathematical Models

Based on the analysis of variance (ANOVA), the influence of temperature, time, and oxalic acid concentration on the amount of reducing sugar was examined in the sample pretreatment. The mathematical model representing the relationship between pretreatment factors and sugar yield was generated and visualized in the 3D model (Figure 3 and Figure 4). It was found that the red-shaded region represents the increasing amount of reducing sugar, which is proportional to the increase of temperature and time. Additionally, it is found that the red-shaded represents the increasing amount of reducing sugar which is proportional to the increasing temperature and concentration of oxalic acid. However, it was observed that the concentration of reducing sugar was not affected by the oxalic acid concentration unless the pretreatment temperature was increased. Hence, oxalic acid concentration alone has no effect on the increase in the concentration of reducing sugars (Figure 2).

A



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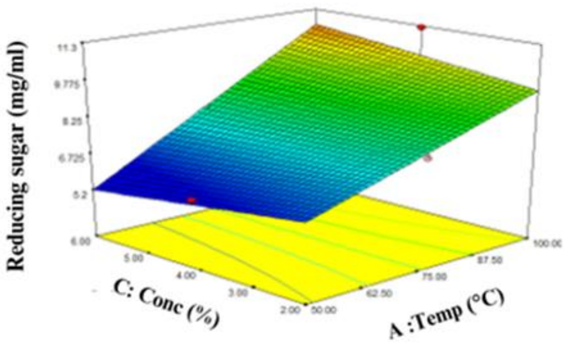


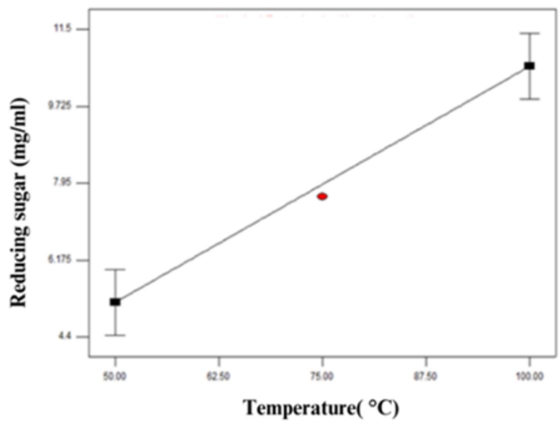
Fig. 3. 3D Response surface plot represents the effect of pretreatment factors on reducing sugar concentration (mg/ml). A; pretreatment time vs pretreatment temperature. B; the oxalic acid concentration vs pretreatment temperature.

The mathematical model and predicted optimal parameters for the pretreatment were simulated by ANOVA analysis, which suggested an oxalic acid concentration of 6%(w/v), pretreated at 100 ºC for 105 min as the optimum pretreatment conditions to obtain 10.646 mg/ml of reducing sugar concentration as a maximum yield according to the predicted model. To validate the prediction, the pretreatment of Napier grass samples was conducted under the optimal pretreatment conditions suggested by the mathematical model (Table 4). To confirm the prediction, the pretreatment of the Napier grass samples was then performed again under the optimum pretreated condition suggested by the mathematical model (Table 4).

Mathematical Model: Final equation in terms of coded factors :

$$\text{Reducing sugar} = +7.18 + (0.95*A) + (1.17*A*B) + (0.75*B^2) \quad (1)$$

A



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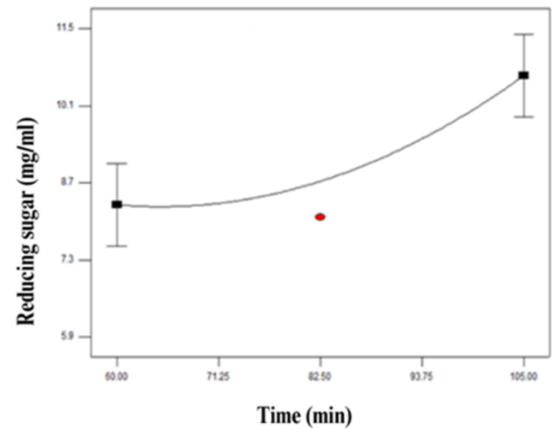

Fig. 4. Relationship between each pretreatment factor, A; pretreatment temperature (ºC), B;Pretreatment time (min) and Y - axis is a concentration of reducing sugar (mg/ml).

Table 4. The optimal condition of pretreatment was obtained from RSM.

Predicted optimal condition			**Reducing Sugar concentration (mg/ml)**		
Temp (°C)	Time (min)	Acid (%,w/v)	Predict	Experimental	%Error
100	105	6	10.65	10.67	0.22

3.4 FTIR Analysis

The FT-IR technique was employed to analyze the chemical structure changes of biomass before and after pretreatment. Figure 5 shows the FT-IR graphs of untreated and pretreated Napier grass. The FT-IR analysis of both untreated and pretreated biomass revealed similar peaks at different intensities across various wavenumbers. In Figure 5, the broad peak between 3000 and 3500 cm^{-1}

corresponds to the O–H stretching vibrations of hydrogen bonds associated with cellulose [30]. The peak around 1627 cm^{-1} indicates the presence of C=C and C=O bonds in the aromatic rings of lignin [31] (Figure 5). These peaks were observed in both the untreated and pretreated biomass; however, their intensities were reduced in the pretreated Napier grass. Another peak at 1034 cm^{-1} is attributed to the C–OH bending in hemicellulose [31,32]. Overall, the FT-IR analysis confirms that the oxalic acid pretreatment resulted in delignification, exposing more cellulose for hydrolysis. Consequently, the FT-IR analysis provides evidence that the pretreatment with oxalic acid effectively delignified the biomass, making more cellulose accessible for hydrolysis.

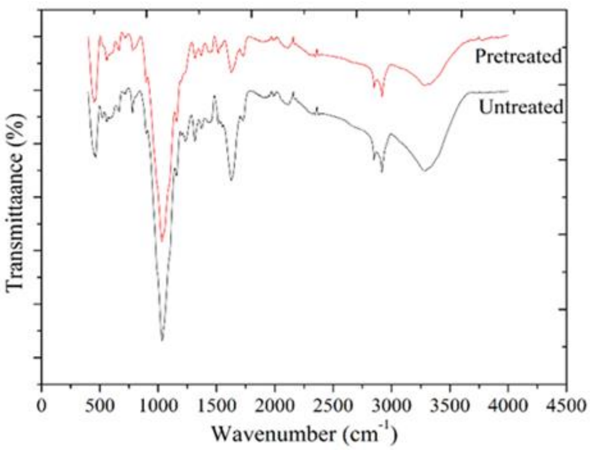

Fig. 5. FTIR analysis of untreated and pretreated of Napier grass.

4 Conclusion

The present study aims to investigate the pretreatment of Napier grass in order to identify the optimal conditions for maximizing the conversion of reducing sugars into biofuel products. This research was carried out to identify the optimum conditions based on the Box - Behnken design of the response surface methodology. The RSM design predicted the maximum yield of reducing sugar concentration with pretreatment using 6% oxalic acid, at 100 °C for 105 min as the optimum pretreatment condition. The Box - Behnken design along with ANOVA analysis predicted 10.65 mg/ml as a maximum yield of reducing sugar concentration when biomass was pretreated at the optimum predicted pretreatment condition. Hence, this research offers valuable insights to future investigators regarding the optimal conditions for pretreating biomass with oxalic acid to maximize sugar yield. In addition to this, further studies can be conducted to recycle oxalic acid from the pretreatment to make the process more economical.

Acknowledgements

The authors would like to thank King Mongkut's University of Technology North Bangkok, Bangkok, Thailand (Grant contract no: KMUTNB-PHD-64-02) for financial support of this work.

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