Optimization of Organosolv Pretreatment with Acid Catalyst to Enhance Enzymatic Saccharification of Corn Husk

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Abstract. Due to awareness of global warming and the devastation of environmental resources, the management of agricultural residues after each harvesting season has been integrated into the biorefining process to increase its value and mitigate environmental pollution caused by burning or combustion. This research focuses on the process development to utilize agricultural biomass residues for renewable energy production in the form of bioethanol. The study employed organosolv pretreatment with sulfuric acid as a catalyst to promote the enzymatic conversion of corn husk into reducing sugars. To determine the optimal conditions for the process, a one-factor-at-a-time method was initially employed to assess the influence of temperature (80-140 ºC), time (40-60 min), and sulfuric acid concentration (0.01-0.5% w/w). Subsequently, Response Surface Methodology (RSM) was conducted based on the Box-Behnken design (BBD) to identify the optimal pretreatment conditions. The predicted optimal pretreatment conditions were found to be 135.4 ºC, 57 min, and 0.46% w/w, resulting in a reducing sugar yield of 20.69% with a margin of error of 1.2%. Additionally, biomass composition analysis and Fourier Transform Infrared spectroscopy (FTIR) were performed to decipher the mechanism of organosolv pretreatment on enzymatic saccharification. This study demonstrated the potential of corn husk as an alternative raw material for the production of value-added products like bioethanol. The obtained reducing sugars serve as vital substrates for the fermentation process required to produce bioethanol as an alternative fuel to meet the target of sustainable development goals (SDGs).

Keyword. Bioethanol, Biorefinery, Enzymatic saccharification, Organosolv pretreatment, Sulfuric acid

1 Introduction

As a result of the global COVID-19 pandemic and the implementation of lockdown measures by several nations in 2020 [1,2], the worldwide production of ethanol experienced a decline. The production volume dropped from 113.7 billion liters in the previous year to 101.7 billion liters, falling short of the target of 114.3 billion liters. This decrease was primarily due to production cuts in countries such as Brazil, the United States, and the European Union. Consequently, ethanol factories reduced fuel ethanol production and instead focused on manufacturing industrial-grade ethanol for cleaning and disinfection purposes. However, the trade volume of industrial-grade ethanol remained relatively small and could not fully substitute the trade of fuel ethanol. Additionally, in 2020, the average retail price for ethanol rose by 5.2% to approximately 23.2 baht per liter, reaching its highest point in the second cycle year (with the average price ranging from 24 to 25 baht per liter) due to the increase in sugarcane and molasses prices as in Figure 1.

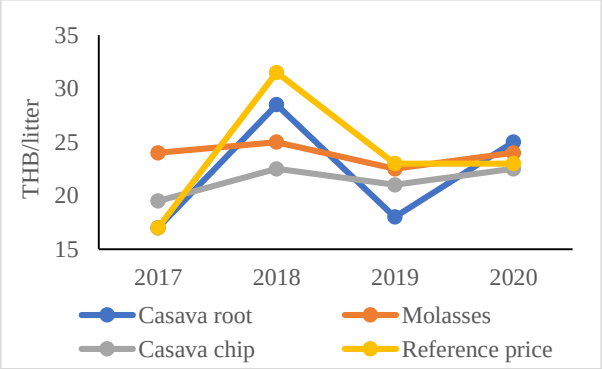

Fig 1. Price chart of ethanol obtained from different raw materials [3].

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The production of industrial-grade ethanol in Thailand has witnessed a significant surge due to the high demand for pure alcohol and hand sanitizers. Production of ethanol in Thailand relies on molasses, cassava, and sugarcane juice as its primary raw materials. As of April 2023, ethanol derived from molasses constituted 65.7% of the total ethanol output, while cassava and sugarcane juice accounted for 28.75% and 5.55%, respectively [4]. Thailand, being rich in agricultural resources, generates substantial amounts of waste materials from its agricultural industry, including straw, rice husks, coconut shells, sawdust, leaf scraps, tree bark, fruit peels, corn cobs, and bagasse. Research conducted earlier has revealed that the agro-industrial sector in Thailand produced an enormous 62 million tons of residual biomass in 2020, which is equivalent to 13,348 thousand tons of crude oil [5]. The amount of this waste biomass material shows the possibility to utilize these biomass residues for the production of alternative energy to increase the energy security of the country. This practice enhances the value of agricultural products, meeting the demands of both local and international consumers. Moreover, it serves the purpose of maximizing the utilization of natural resources, thereby minimizing agricultural waste and reducing pollution [6].

Corn is a significant economic crop grown in various regions of Thailand, but its husks are not widely utilized compared to other agricultural waste. These wastes are often burned directly to generate heat and electricity. This research focuses on assessing the potential of corn husks as a source of lignocellulosic biomass for the production of bioethanol. The husks contain abundant cellulose and hemicellulose, which can be hydrolyzed into monomeric sugars and converted into ethanol. The bioethanol production process typically involves four essential stages: pretreatment, hydrolysis, fermentation, and product recovery/extraction [7, 8]. Due to the natural recalcitrant structure of lignocellulose, the efficiency of the cellulase enzyme that hydrolyzes biomass to sugars is low [9,10]. To improve the enzymatic hydrolysis, pretreatment is necessary. Pretreatment can be performed in several ways, such as physical, chemical, biological, and physicochemical pretreatment [11,12], each of which is suitable for different applications. However, these pretreatment methods affect the arrangements of lignocellulose-type materials, which increases the surface area and porosity, which is beneficial to enzymatic digestion [13,14]. After pretreatment, cellulose components are subjected to enzymatic hydrolysis to produce sugars that are subsequently converted to value-added products, such as biofuels, biochemicals, platform chemicals, and biopolymers [15, 16, 17].

Organosolv solutions are used in the pretreatment of lignocellulose to remove lignin, inhibitors of cellulase, and reorganize the lignocellulose structure, ultimately enhancing the efficiency of bioethanol production [18]. The most commonly used solvents for lignin extraction are acetic acid, formic acid, ethanol and glycerol [19]. In general, organosolv solutions have been applied in the production of kraft and alkali lignin from the fiber pulp industry to obtain high-purity lignin. Several organosolv solutions are considered to be green solvents as they are derived from natural sources and are environmentally friendly chemicals. Glycerol is one of the solvents to be used in organosolv pretreatment. Crude glycerol is produced as a by-product of biodiesel production with low purity [20]. Therefore, using glycerol is a way to meet the circular economy concept.

Previously, the pretreatment of rice straw using the organosolv method was investigated. Glycerol was utilized as the solvent with a concentration of 70% in the reactor, and the process was carried out at a temperature of 220 ℃. The researchers employed an X-ray diffraction (XRD) technique to examine the structural changes in lignocellulose, and the results demonstrated that glycerol pretreatment effectively reorganized the cellulose structure. The amorphous components, including lignin and hemicellulose, were effectively removed during the process which could potentially enhance the efficiency of hydrolysis, as observed through the use of infrared wavelength and chemical structural analyzers. These tools identified crucial bonding structures, such as β-ethers, β-esters, and hydrogen bonds [21]. Similarly, the pretreatment of sugarcane bagasse with Lewis acid-mediated glycerol (80 wt%) combined with 1.0 wt% AlCl3 was studied. Under pretreatment conditions of 150 ℃ for 1 hour, excellent results were obtained with a glucan recovery rate of 98.47%, xylan removal rate of 95.31%, and delignification rate of 49.69%. The efficiency of acidified glycerol pretreatment remained relatively stable during four consecutive cycles, as demonstrated in reutilization experiments. When subjected to in situ enzymatic saccharification after pretreatment, the bagasse yielded approximately 50% glucose at a glycerol concentration of 16 wt%. Following pretreatment, the lignin was solubilized in the pretreated hydrolysate, resulting in two types of lignin: organosolv lignin (OL) and cellulolytic enzyme lignin (CEL), present in the solubilized form and the solid residue, respectively. Further examination of the structure and molecular weights of these two lignin samples revealed their significant potential for being converted into valuable products [22].

Hence, this study aims to optimize the pretreatment method by using a combination of glycerol and acid catalyst to convert corn husks to sugars. The optimization experiments were designed using the Response Surface Methodology (RSM) with Box-Behnken design to identify pretreatment parameters and levels promoting the maximum conversion of corn husk to sugars. This approach aims to add value to the corn husks and minimize environmental pollution, rather than allowing them to be decomposed or wasted. This optimization process is conducted to facilitate bioethanol production from the corn husks by the biorefining process.

2 Material and methods

2.1 Raw materials

Corn husks were obtained from urban markets in Bangkok, Thailand. Corn husks were washed with distilled water and dried at 60 ºC for 12 h in a hot air oven

to remove moisture. After drying, it is crushed using a grinder and filtered through an aluminium sieve of 40 mesh and 20 mesh, respectively to obtain a uniform sample. The biomass was kept in a plastic bag, tightly sealed, and stored at room temperature until it was needed for future use. Commercial cellulase Ctec2 from *Trichoderma reesei* was purchased from Sigma-Aldrich (aqueous solution of 700 units /ml).

2.2 Organosolv pretreatment

Pretreatment was performed by soaking 3 g of ground corn husk in 30 g of glycerol solution mixed with sulfuric acid at a solid loading ratio of 10% w/w. The treatment was carried out in a hot air oven. Heat treatment using 70% glycerol concentration mixed with sulfuric acid (at different concentrations from 0.01% to 0.5% w/w), temperature (80 to 140 ℃) and time (40 to 60 minutes). Following the pretreatment process, the liquid fraction and solid residue were separated using a Whatman No. 1 filter paper through filtration. The solid residue underwent additional washing with distilled water until reaching a neutral pH. Subsequently, the solid was dried in a hot air oven at 60°C for a duration of 8 hours. The treated samples were then stored in a desiccator in preparation for the subsequent enzymatic saccharification step.

2.3 RSM Experiment design

To determine the ideal pretreatment conditions (X) for maximizing sugar yield (Y), an optimization experiment using the Box-Behnken Design (BBD) with Response Surface Methodology (RSM) was conducted. Experimental data was collected and analyzed using Design Expert software version 7.0.0. Three factors, namely pretreatment temperature (X_1), time (X_2), and sulfuric acid concentrations (X_3), were varied across three levels as shown in Table 1. The pretreatment process was carried out within the ranges of 80 - 140 ℃ (X_1), 40 - 60 minutes (X_2), and sulfuric acid concentrations of 0.01%wt - 0.5%wt (X_3). Each factor was assigned levels of -1, 0, and +1 as indicated in Table 1. A total of 17 experimental runs were conducted according to the RSM design outlined in Table 2.

Table 1. Independent variable factors in pretreatment condition of corn husk on RSM.

Independent variable factors	Coded symbols	Levels		
		-1	0	-1
Temperature (°C)	X_1	80	139.66	140
Time (min)	X_2	40	44.27	60
Sulfuric acid concentration (%wt)	X_3	0.01	0.41	0.50

After pretreatment, the treated biomass underwent hydrolysis using cellulase enzymes, and the resulting liquid hydrolysates were analyzed to determine the quantity of reducing sugars produced. The amounts of reducing sugars obtained from each experiment were subjected to analysis of variance (ANOVA) to establish the correlation between the pretreatment parameters (model p-value < 0.05). Through multiple regression analysis, the optimal pretreatment conditions that would yield the highest sugar content were predicted. The generated second-order model was evaluated based on the coefficient of determination (R^2), the significance of the test conditions in the ANOVA, and the lack of fit test.

Table 2. RSM experimental design for organosolv pretreatment of corn husk

Run	Pretreatment condition			Sugar yield (%)
	X1: Temperature (°C)	X2: Time (min)	X3: Sulfuric concentration (%w/w)	
1	110.00	50.00	0.26	13.41
2	110.00	40.00	0.01	6.45
3	140.00	50.00	0.50	19.88
4	80.00	40.00	0.26	4.14
5	140.00	50.00	0.01	5.95
6	110.00	60.00	0.01	5.32
7	80.00	60.00	0.26	9.00
8	110.00	50.00	0.26	12.94
9	110.00	60.00	0.50	15.37
10	80.00	50.00	0.01	4.46
11	110.00	50.00	0.26	11.62
12	140.00	40.00	0.26	18.32
13	140.00	60.00	0.26	19.38
14	80.00	50.00	0.50	8.56
15	110.00	50.00	0.26	12.21
16	110.00	40.00	0.50	12.07
17	110.00	50.00	0.26	13.74

2.4 Enzymatic hydrolysis

Commercial Ctec2 was used to enzymatically hydrolyze both untreated and pretreated corn husks. The hydrolysis reaction was conducted with 0.5 g of biomass in 20 mL of 50 mM citrate buffer, supplemented with 200 µl of 2 M sodium azide solution and 10 µl of Cellic CTec2 (20 FPU/g-biomass) [23,24,25]. The reaction mixture was then incubated at 50 °C and 150 rpm for a duration of 72 hours [6]. The mixture was incubated at 100 ℃ for 10 minutes to stop the reaction. The liquid hydrolysate fraction was obtained by centrifuging the mixture at 6000 rpm for 10 minutes. The concentrations of reducing sugars in the liquid hydrolysate were then determined using the dinitrosalicylic acid (DNS) method [26,27].

In brief, 50 mL of the enzymatic hydrolysis supernatant was mixed with 150 mL of the DNS solution. The mixture was then incubated in a water bath at 95℃ for 5 minutes. The mixture was subsequently placed on ice for 5 minutes to stop the reaction. After incubation, 1 mL of distilled water was added to the mixture and thoroughly mixed using a vortex shaker. The absorbance of each sample was measured at 540 nm using a UV/VIS spectrophotometer. The concentration of reducing sugar was determined by comparing the absorbance values to a glucose standard curve, which established the correlation between sugar concentration and absorbance.

2.5 Composition analytical methods

The compositions of corn husk, including cellulose, hemicellulose and lignin before and after pretreatment were determined by using the Van Soet method [28,29].

The chemical structures and chemical bonding arrangements of corn husk samples were analyzed by Fourier transform infrared analyzer (FTIR) (Nicolet Is50) by scanning the wavenumbers between 400-4000 cm^{-1}.

3 Result and discussion

3.1 Screening of pretreatment parameters by OFAT method

Before RSM optimization, the tested ranges of pretreatment parameters were determined by using the one factor at a time (OFAT) method. Preconditioning with different pretreatment temperatures was varied at 80, 110 and 140 ℃, while using a constant sulfuric acid concentration of 0.1 wt% and a pretreatment time of 20 min. After the range of pretreatment temperature was screened and selected, the concentration of sulfuric acid was tested at 0.02, 0.1 and 0.5%wt, while the pretreatment temperature and pretreatment time were fixed at one condition. Lastly, after the selection of the ranges of pretreatment temperature and acid concentration, the pretreatment time was varied at 20, 40 and 60 min, respectively.

In Figure 2, the results clearly showed that the yield of the reducing sugar at 80 ℃ has the highest yield of reducing sugar (13.72 g per 100 g corn husk). For the pretreatment time, the highest yield of reducing sugars at 10.22 g per 100 g corn husk was obtained at 60 min of pretreatment time. Furthermore, the maximum yield of reducing sugar at 22.10 g per 100 g corn husk was obtained when using 0.5%wt of sulfuric acid concentration.

3.2 Optimization of pretreatment based on RSM

RSM is used for determining the optimum state for independent factors (i.e. pretreatment factor) to obtain the minimum or maximum points of dependent factors (i.e. sugar yield). Furthermore, it assesses the impact of each independent variable and its interactions with the RSM-dependent variable, minimizing the need for excessive experimental trials, thus saving time and reducing trial costs. Additionally, RSM has the capability to predict outcomes and generate surface models based on various conditions of the independent variables. Several studies have applied RSM to optimize and develop processes in various areas [30,31,32,33].

In this study, after 17 runs of pretreatments were conducted by using the condition shown in Table 2, the reducing sugar yield obtained from all runs was analyzed by using ANOVA (Table 3). The predicted model was suggested to be the second-order model with a p-value of 0.0012 suggesting the model significance (p-value < 0.05). Also, the R^2 value of the predicted model was determined to be 0.9456 indicating a low variance between experiments and demonstrating the reliability of the model. Generally, an R^2 value above 0.9 is considered significant [34].

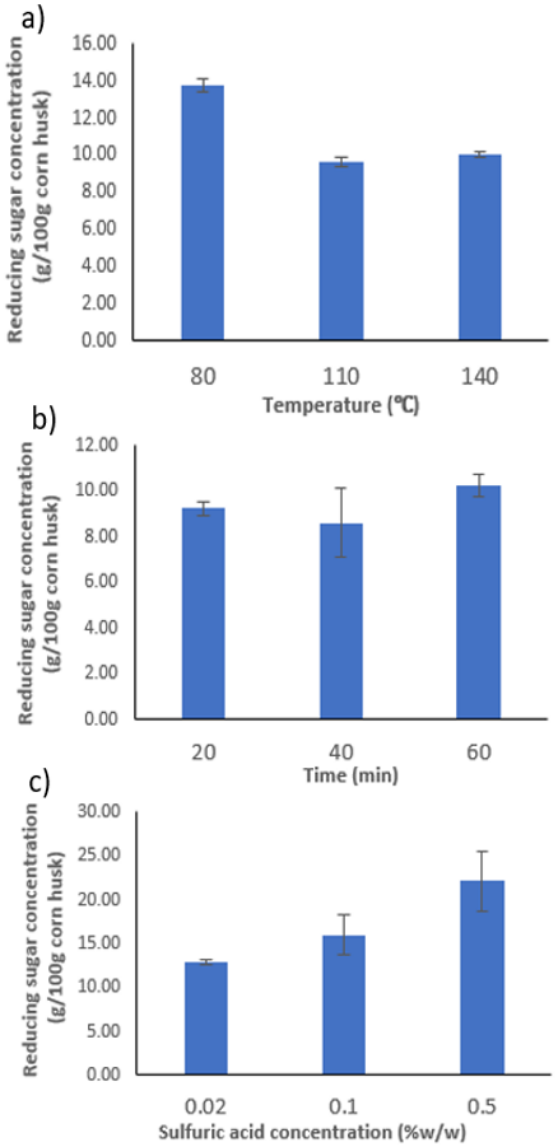

Fig 2. Effect of (a) pretreatment temperature, (b) time and (c) sulfuric acid concentration on the reducing sugar yields based on OFAT method.

Table 3. Analysis of variance (ANOVA) for the predicted model of pretreatment optimization. The significance of each parameter was justified at a p-value < 0.05.

Source	Squares	df	Mean square	F Value	p-value
Model	395.29	9	43.92	13.53	**0.0012**
A-Temp	174.55	1	174.55	53.76	**0.0002**
B-Time	8.16	1	8.16	2.51	0.1568
C-conc	141.92	1	141.92	43.71	**0.0003**
AB	3.62	1	3.62	1.12	0.3259
AC	24.15	1	24.15	7.44	**0.0295**
BC	4.91	1	4.91	1.51	0.2587
A^2	0.03	1	0.03	0.01	0.9267
B^2	0.00	1	0.00	0.00	0.9929
C^2	37.62	1	37.62	11.59	**0.0114**
Residual	22.73	7	3.25		
Lack of Fit	19.70	3	6.57	8.67	0.0318
Pure Error	3.03	4	0.76		

From the ANOVA analysis of the model (Table 3), it was found that the F value was 13.53 with statistical significance (p-value < 0.05) and there was a 0.01% chance of interference of themodel F value. Pretreatment parameters, such as temperature and the concentration of sulfuric acid were the important normalization factor according to the ANOVA analysis. The effect of each pretreatment factor, temperature, time, and sulfuric acid concentration on sugar yield was visualized as shown in Figure 4. Furthermore, the interaction effects of two pretreatment factors at a time were observed in the form of 3D-contour plots (Figure 5). The data showed that the yield of the reducing sugars increased when temperature, time and acid concentration were increased. This observation could be explained that this pretreatment condition may lead to swelling in the cellulose structure and a change in the structural organization of hemicellulose and lignin, which leads to an increase in the surface area of biomass to cellulase. As a result, this allows the enzymes to have better access to cellulose and reactivity leading to increased sugar production accordingly [35,36].

From the ANOVA analysis, the generated mathematical model predicted the optimal levels of pretreatment parameters to be at 135.46 ℃ for 57 min and at a sulfuric acid concentration of 0.46 (%w/w) to achieve the maximum sugar yield at 20.69% (Table 4). To confirm the reliability of this predicted model, the pretreatment experiment was performed again at the predicted optimum pretreatment conditions. The sugar yield obtained from the experiment was 20.43% with a deviation of only 1.2% from the predicted model value. When comparing the yield of reducing sugar, it can be inferred that the model from ANOVA prediction has high accuracy.

Table 4. Optimal pretreatment condition obtained from RSM experiment.

Optimal pretreatment parameter		
Temperature(°C)	Time (min)	Sulfuric acid concentration (%w/w)
135.4	57	0.46
Predicted sugar yield (%)	Experimental sugar yield (%)	Difference (%)
20.69	20.43	1.2

The effects of pretreatment factors for the organosolv method in this study were in good agreement with the previous studies. Razi *et. al.,* (2020) studied the organosolv pretreatment of peanut shells under various conditions of time, biomass loading and sulfuric acid concentration using Central Composite Design in RSM to determine the correlation of the variables associated with lignin content after pretreatment [37]. Izzi *et. al.,* (2020) conducted sodium hydroxide pretreatment using under reaction time, temperature, and concentration based on BBD in RSM [38]. This study reported a similar relationship between the content of the reducing sugar and the pretreatment parameters. It was found that these parameters significantly correlated with the reduction of sugar.

3.3 Effect of organosolv pretreatment with acid catalyst on the chemical composition of corn husk.

To understand the mechanism of how glycerol with acid can enhance enzymatic saccharification, the compositions of untreated and pretreated corn husks were analyzed (Table 5). The cellulose content of corn husk was increased from 36% to 77.5% after pretreatment with optimal conditions at 135.46 ℃ for 57 min and at a sulfuric acid concentration of 0.46 (%w/w). The hemicellulose content of corn husk decreased after pretreatment from 49.7% to 4.6%. Additionally, lignin content was removed from biomass due to organosolv pretreatment. Due to these changes in lignocellulose compositions, the hemicellulose and lignin were removed allowing more accessibility of cellulose to cellulase [39,40]. Therefore, the release of reducing sugars of pretreated biomass was increased during enzymatic saccharification.

Table 5. Composition analysis of pretreated corn husk as an optimal parameter and untreated corn husk.

Sample	Untreated	Pretreated
Cellulose (%)	36	77.5
Hemicellulose (%)	49.7	4.6
Lignin (%)	3.07	8.87

3.4 FTIR characterization of the raw and pretreated samples.

To observe the influence of pretreatment on the chemical arrangement and bonding, FTIR analysis was done by using the wave number range 40-4000 cm^{-1}. The Infrared absorption spectra were shown in Figure 3. The stretching mode of the OH bond in cellulose, representing the chemical bond, was observed as peak intensity within the range of 3300 cm^{-1} to 3600 cm^{-1}. Additionally, signals in the wave number range of approximately 2800-2900 cm^{-1} were detected, corresponding to the stretching mode of CH bonds in cellulose [38]. In the wavelength range of 1715-1765 cm^{-1}, the presence of C=O groups in hemicellulose was identified [41]. The organosolv pretreatment effectively dissolved hemicellulose, as evidenced by the peak at 1100-1000 cm^{-1} corresponding to the bending of the C-OH functional group in hemicellulose disappeared [41]. Moreover, a peak observed at 1032 cm^{-1} indicated the C-O vibration of carbon C_2, while the 1632 cm^{-1} band represented carbonyl groups [41]. The presence of lignin was detected in the natural state within the wavelength range of 1315-1942 cm^{-1}. Comparatively, the pretreated corn husk exhibited lignin at this wavelength, distinguishing it from the untreated corn husk. Altogether, the FTIR results were well agreed to composition analysis (Table 5).

4 Conclusion

This study demonstrates the potential of corn husk as a feedstock for biorefining processes. It also provides an

alternative to reducing agricultural waste and air pollution. The maximum yield of reducing sugars was obtained from optimally pretreated corn husks by the organosolv method. These sugars have the potential to be converted into various products, such as biogas and bioethanol. Initially, the pretreatment process was conducted using the one factor at a time method to determine the optimal range for testing the pretreatment conditions. Then, the pretreatment condition was optimized by using RSM. The predicted condition of pretreatment was suggested to be at a temperature of 135.4 ℃, a time of 57 min and a sulfuric acid concentration of 0.46 (%w/w). The yield of reducing sugar was 20.69%, which was the highest yield of reducing sugar under the best conditions.

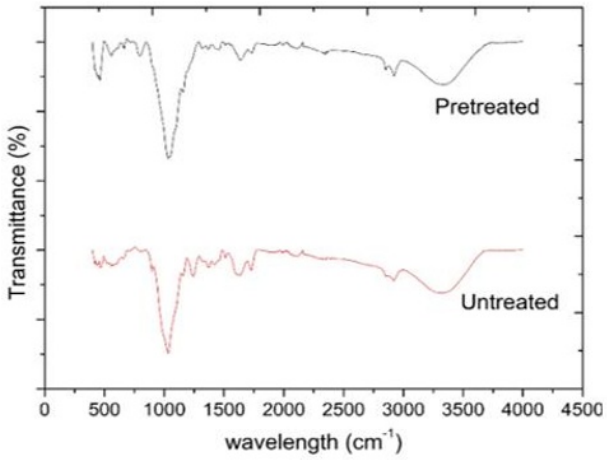

Fig. 3. FTIR spectra of untreated and pretreated corn husk.

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