Research Article

Influence of Sulfuric Acid Pretreatment and Inhibitor of Sugarcane Bagasse on the Production of Fermentable Sugar and Ethanol

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Abstract
Improper disposal of agricultural waste after harvesting season has posed serious health and environmental issues. Alternative methods to utilize agricultural waste to produce a value-added product, especially biofuel, have become the focus of research and industrial stakeholders. To make the process feasible, the maximum conversion should be achieved with the optimum operational condition. This research applied Response Surface Methodology (RSM) with the Box-Behnken design (BBD) to optimize sulfuric acid pretreatment of sugarcane bagasse by varying three pretreatment factors namely, acid concentration (0.5–3.5%), temperature (60–140°C), and time (20–100 min). Pretreated biomass was enzymatically hydrolyzed, and the effectiveness of pretreatment was examined according to the reducing sugar concentration. However, inhibitors namely, acetic acid, 5-hydroxymethylfurfural (5-HMF), and furfural were produced during pretreatment, which was analyzed through GC-MS analysis. The Box-Behnken design could optimize and correlate the effect of pretreatment parameters on the hydrolysis of sugarcane bagasse. The optimum pretreatment condition was predicted at an acid concentration of 3.50%, the temperature of 136.08°C, and the time of 75.36 min to obtain the maximum sugar production. Sugarcane bagasse pretreatment at optimum condition could produce a reducing sugar of 180.15 mg/g-sugarcane bagasse, which is 3.06 folds higher than untreated sugarcane bagasse. However, ethanol yield from pretreated biomass was less than unpretreated biomass because of the inhibitor formation. This study provides a new insight into utilizing agricultural waste in a more efficient and eco-friendly manner.

Keywords: Bioethanol, Acid pretreatment, Optimization, Fermentation, Reducing sugar, Response surface methodology

1 Introduction

Agricultural waste utilization could be one of the solutions for inappropriate agro-waste combustion leading to severe health issues and environmental pollution [1], [2]. Sugarcane bagasse is one of the most common agricultural wastes produced in Thailand. This biomass waste can be converted into several products such as biofuels, absorbents, insulators, briquettes, medicines, food substances, platform chemicals, and biotechnological materials [3]–[7]. Direct utilization of this agricultural waste is impossible due to the recalcitrant nature of the biomass. Cellulose, hemicellulose, and lignin are the main components in the plant cell wall that are united to form the complex structure of lignocellulosic biomass [8]. These components and their arrangements lead to the recalcitrance of biomass. However, the ratio of each component might be varied according to the species of plants. For instance, a higher amount of hemicellulose exists in wheat straws and leaves, while much quantity of cellulose is displayed in hardwood [9]. Furthermore, different ages, stages of growth, and other factors can also affect the amount of each component in single plant species [10].

In the plant cell wall, cellulose is the main structural component composed of the linear polymer of D-glucose connected to others by β-(1,4)-glycosidic bonds [11]. These long-chain polymers can be oriented to form cellulose microfibrils [12]. Microfibrils are arranged together to form cellulose fibrils. These cellulose fibrils, arranged along with hemicellulose and lignin, are resistant to enzymatic hydrolysis [13]. In addition to this, cellulose can be present in plants in its crystalline form and amorphous form. Mostly, cellulose appears in its crystalline form, whereas a small amount will occur as an amorphous form, which is more susceptible to enzymatic hydrolysis [14],[15]. Hemicellulose, representing 20–35% of biomass is a heteropolymer containing various monosaccharides like xylan, mannan, glucomannan, etc. Hemicellulose is amorphous material [13]. Cellulose and hemicellulose are linked together in biomass by hydrogen bonds. Lignin is another component in biomass, comprising about 15–40% of dry weight. It is also a heteropolymer, which is amorphous and composed of p-coumarin, coniferyl, and sinapyl alcohol. Lignin provides structural rigidity and helps in binding hemicellulose to cellulose in the cell wall [13].

With this complex structure of biomass and with the strong interaction between its components, utilization of biomass without any prior pretreatment becomes difficult. Hence, pretreatment is recognized as a necessary process for converting biomass. The major objectives of pretreatments are disintegrating lignin structure, decreasing the crystallinity of hemicellulose and cellulose, and enlarging the porosity of the lignocellulose to allow acids or enzymes to enter and hydrolyze cellulose [16]. Pretreatment could be fundamentally classified into different categories as follows; physical (milling or grinding) [17], physicochemical (autohydrolysis or hydrothermolysis) [17], chemical (alkali, dilute acid, oxidizing agents and organic solvents, ionic liquid) [18]–[23], biological [24]–[27] and electrical [28].

The most preferred pretreatment is acid pretreatment where biomass is either pretreated with diluted acid or concentrated acid. Concentrated acid is extensively used as a pretreatment agent, but it should be used cautiously for applications because of its toxicity, corrosiveness, and hazardous nature [29]. Diluted acid hydrolysis has been applied in lignocellulosic biomass pretreatment, for instance, diluted sulfuric acid (< 4 wt%) was utilized as a prudent and productive solvent for the cellulosic biomass industry [30]. Diluted sulfuric acid pretreatment potentially catalyzes the reaction rates and helps in cellulose hydrolysis [31]. Furthermore, it can hydrolyze and digest hemicellulose to be xylose and small molecule of sugars [32]. Diluted acid pretreatment with high temperature has the capability in cellulose hydrolysis [33]. Diluted acid hydrolysis is commonly conducted at high temperatures, whereas concentrated acid hydrolysis uses low temperatures [34]. However, hydrolysis of biomass by acid can also lead to degradation of sugars to form inhibitors like furfural, 5-HMF. These inhibitors could decrease the sugar yields [35].

Pretreatment is followed by enzymatic hydrolysis to disintegrate the cellulose and hemicellulose completely into proper monomers (e.g. sugars) so that microorganisms can help in the conversion of lignocellulosic biomass to products. Consequently, the sugar monomers could be transformed into diverse value-added products of biofuels such as biodiesel, bioethanol, biomethane, and butanol based on microorganisms applied in the fermentation process.

In this experiment, diluted sulfuric acid (H_2SO_4) was used for the pretreatment of sugarcane bagasse to enhance enzymatic saccharification of lignocellulosic biomass. Being the most used chemical for the pretreatment of biomass due to its low cost and efficiency in lignin removal, diluted sulfuric acid was chosen in this study for pretreating sugarcane bagasse. Moreover, Response Surface Methodology (RSM) was used to optimize the pretreatment conditions and escalate the amounts of reducing sugars, which could be further converted to bioethanol in downstream processing. Fourier Transform Infrared Spectrophotometer (FTIR) was used for analyzing the chemical structures of pretreated biomass. The advantage of this technique is that it requires a short time for measurement and is safe for both liquid and solid samples [36]. Also, this study could utilize and convert the sugarcane bagasse to be bioethanol and alleviate environmental issues caused by the inappropriate combustion of biomass waste.

2 Materials and Methods

2.1 *Preparation of raw material*

Sugarcane bagasse was obtained from a sugar factory in Thailand (Courtesy provided by KTIS bioethanol co. Ltd). It was processed by the factory and then transferred to the laboratory in the form of chopped and dried samples. The sugarcane bagasse was ground into powder using a household blender and later stored in an airtight container at room temperature for further use.

2.2 *Experimental design*

Box Behnken Design of Response surface methodology (RSM) was used to determine the optimum pretreatment condition that provides maximum reducing sugar yield (Y). The design has considered three factors for optimization. These factors include pretreatment temperature (X_1) varying from 60–140°C, pretreatment time (X_2) varying from (20–100 min), and acid concentration (X_3) ranging from 0.5–3.5%. Each pretreatment factor was adjusted to three levels, i.e., high (+1), mid (0), and low (–1). A total of 17 runs were carried out by varying each factor. The data was analyzed by Design-Expert Version 7.0 software. Table 1 represents the design of the experiment with various pretreatment conditions applied for the study. The pretreated biomass was further used for enzymatic hydrolysis [37].

Table 1: Box-Behnken design for pretreatment of sugarcane bagasse with different pretreatment conditions

Run	Pretreatment Condition			Concentration of Reducing Sugar (mg/mL)
	Temp. (°C)	Time (mins)	Conc. of H_2SO_4 (%)	
	X_1	X_2	X_3	Y
1	140	100	2	4.774
2	100	20	3.5	1.917
3	60	60	3.5	1.418
4	100	60	2	2.788
5	60	20	2	1.157
6	100	20	0.5	1.428
7	140	60	0.5	3.215
8	100	60	2	3.056
9	60	100	2	1.39
10	100	60	2	3.079
11	140	60	3.5	4.328
12	100	100	0.5	2.788
13	60	60	0.5	1.193
14	140	20	2	2.128
15	100	60	2	2.977
16	100	60	2	2.798
17	100	100	3.5	4.131

2.3 *Enzymatic hydrolysis*

The pretreated biomass was enzymatically hydrolyzed to further determine the reducing sugar from it. For the hydrolysis, 100 mg of biomass was added into 4 mL of 0.05M citrate buffer (pH 4.8). Sodium azide (2 M) was also added to avoid any microbial contamination in the hydrolysis solution. CelluClast 1.5 L (35 µL) and β-glucosidase (10 µL) enzyme were added, and the reaction mixture was incubated at 45°C and 150 rpm for 72 h. The reducing sugar was analyzed according to the standard dinitrosalicylic acid (DNS method) proposed by Miller [38]. The effect of the pretreatment factor on the amount of released reducing sugar was statistically analyzed by ANOVA using the SPSS program (Version 26.0).

2.4 *Inhibitor analysis*

Potential inhibitors produced during pretreatment of biomass were analyzed using Gas Chromatograph-Mass Spectrometer (GC-MS). The sample was analyzed for furfural, hydroxymethylfurfural (HMF),

and acetic acid using Shimadzu GCMS equipped with DB-Wax column (Agilent). The sample was analyzed using Helium as a carrier gas with a total flow of 41.8 mL/min and a column flow of 1.25 mL/min. GC inlet was set in split mode with a split ratio of 30. The column oven temperature and injector temperatures were 50°C and 250°C respectively. The temperature program was set from 50°C (1 min hold time) to 120°C (2 min hold time). The temperature was again increased to 170°C (1 min hold time) and finally raised to 240°C (10 minutes hold time). The MS program was set with ion source temperature at 200°C and the mass range was from m/z 40 to 600.

The sample was analyzed for HMF using Helium as a carrier gas with a total flow of 41.8 mL/min and a column flow of 1.25 mL/min. GC inlet was set in split mode with a split ratio of 30.0. The column oven temperature and injector temperatures were 50°C and 250°C respectively. The temperature program was set from 50°C (2 min hold time) to 110°C and finally raised to 250°C with 15 min hold time. The MS program was set with ion source temperature at 200°C and the mass range was from m/z 40 to 600.

2.5 *Fourier Transform Infrared Spectroscopy (FTIR) analysis*

FTIR analysis was carried out on both untreated and pretreated sugarcane bagasse using FTIR spectrometer (Spectrum 2000, Perkin Elmer, USA), with a resolution of 4 cm^{-1} from 400 cm^{-1} to 4000 cm^{-1}. Spectrum 2.00 software was used to analyze the spectral data.

2.6 *Biomass composition*

The biomass composition of the untreated and pretreated biomass was determined as described by Goering and Van Soest [39]. The amount of cellulose, hemicellulose and lignin in the sample was calculated.

2.7 *Fermentation studies*

Fermentation studies were carried out using *Saccharomyces cerevisiae* TISTR 5606 to study ethanol production from sugarcane bagasse. The biomass hydrolysate was prepared from pretreated and untreated sugarcane bagasse according to the enzymatic saccharification protocol eliminating sodium azide addition. The culture media (pH 5.0) comprising of 5% (w/v) sucrose, 1% (w/v) yeast extract, and 19 mL biomass hydrolysate was inoculated with 1 mL of a yeast inoculum. The setup was incubated in a batch at 32°C for 60 h at 150 rpm in a rotary shaker. The yeast culture was centrifuged at 8000 rpm for 10 min to collect the supernatant fraction for analysis of ethanol yield.

2.8 *Determination of ethanol concentration*

The spectrophotometric determination of ethanol concentration was performed as described in previous reports [40]. Briefly, ethanol was extracted from the sample using Tri-n-butyl phosphate (TBP, Sigma Aldrich, USA). For this purpose, 1 mL of TBP was mixed with a 1 mL liquid sample by vortexing vigorously for 1 min. The mixture was separated into two phases by centrifugation at 3420 g for 5 min. The clear, transparent upper phase was the TBP layer, whereas the turbid lower layer was the water. Then, 500 µL of the TBP layer was aspirated to a new microtube and mixed with 500 µL dichromate reagent composed of 10% (w/v) $K_2Cr_2O_7$ in 5 M H_2SO_4. The mixture was vortexed for 1 min and allowed to settle for 10 min at room temperature. This allows the oxidation product present in the lower phase to turn to blue-green color. The optical density was measured after diluting 100 µL oxidation product in 900 µL deionized water. A UV/Vis spectrophotometer (T80+ UV/Vis Spectrometer, PG Instrument Ltd., USA) was used to measure the absorbance of the sample at 595 nm. The concentration of ethanol was calculated using the ethanol standard curve.

3 Results and Discussion

3.1 *Relation between pretreatment parameters and reducing sugar concentration*

Numerous scientific papers have used RSM for determining the pretreatment factors and condition ranges to suitably optimize their experiments [41]–[45]. Also, RSM is usually used for prediction and in empirical modeling [46]. Optimization of the pretreatment conditions was carried out using the Box-Behnken model by considering three factors: temperature, time, and concentration of acid. The

Box-Behnken design used for the experiment and the concentration of reducing sugar obtained at various runs is represented in Table 1. The maximum reducing sugar concentration obtained from these experiments was 4.774 mg/mL in Run No. 1, when the biomass was pretreated with 2.0% H_2SO_4, at 140°C for 100 min.

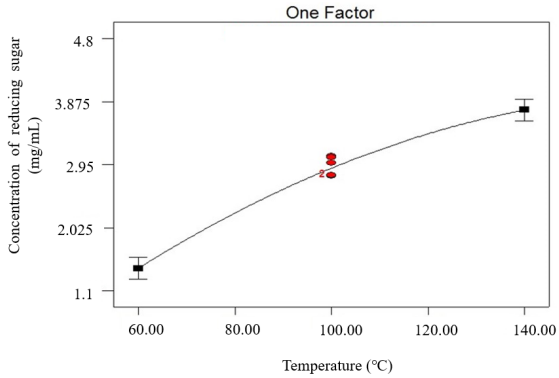
The statistical analysis for the selection of representative model suggested that the correlation model between pretreatment factors and reducing sugars of sugarcane bagasse was a Quadratic model with correlation efficiency (R^2) as 0.9896, which significantly supported the model fitting. Independent (X value) and dependent (Y value) factors in the RSM table were further examined as fitness to the suggested model by using ANOVA analysis. To evaluate the significance of this experimental design, ANOVA analysis was carried out and the data was shown in Table 2. The values in Table 2 clearly showed that the model is statistically significant with p-value < 0.01. This ensures that the model can be used to represent influence of pretreatment parameters on reducing sugar yield. Likewise, according to the Lack of fit test, the p-value obtained was 0.2259 representing to insignificant Lack of Fit model. The p-value for each parameter tested was also less than 0.001, indicating their statistical significance in pretreatment to produce reducing sugars.

Table 2: ANOVA results of response surface reduced quadratic model

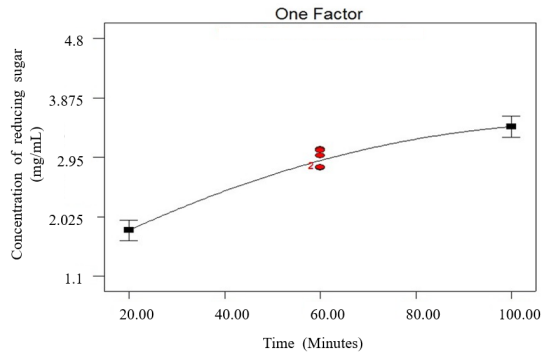
Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F
Model	19.85	8	2.48	79.06	< 0.0001*
A-Temp	10.78	1	10.78	343.42	< 0.0001*
B-Time	5.21	1	5.21	165.92	< 0.0001*
C-Conc.	1.26	1	1.26	40	0.0002*
AB	1.46	1	1.46	46.38	0.0001*
AC	0.2	1	0.2	6.27	0.0367*
BC	0.18	1	0.18	5.81	0.0425*
A^2	0.4	1	0.4	12.73	0.0073*
B^2	0.33	1	0.33	10.54	0.0118*
Residual	0.25	8	0.031		
Lack of fit	0.17	4	0.043	2.25	0.2259
Pure Error	0.077	4	0.019		
Cor Total	20.11	16			

*statistically significant with p-value < 0.05

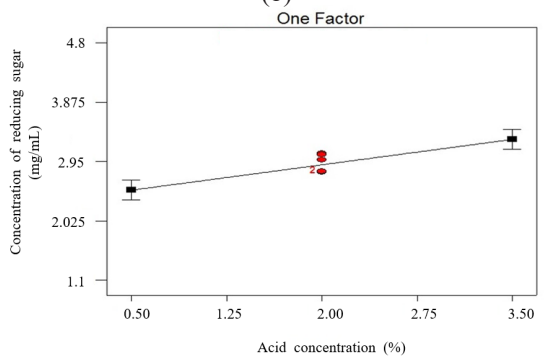
According to the ANOVA analysis, the effects of each pretreatment parameter on the yield of reducing sugar could be predicted by a fit model and can be explained by one coordinating factor plot (Figure 1) and contour plot (Figure 2). The relationship between pretreatment factors (pretreatment temperature, pretreatment time, and acid concentration) and reducing



(a)



(b)



(c)

Figure 1: The relationship between each pretreatment factor, including (a) pretreatment temperature (°C), (b) pretreatment time (min), and (c) acid concentration (%) and reducing sugar concentration (mg/mL) obtained from pretreated sugarcane bagasse.

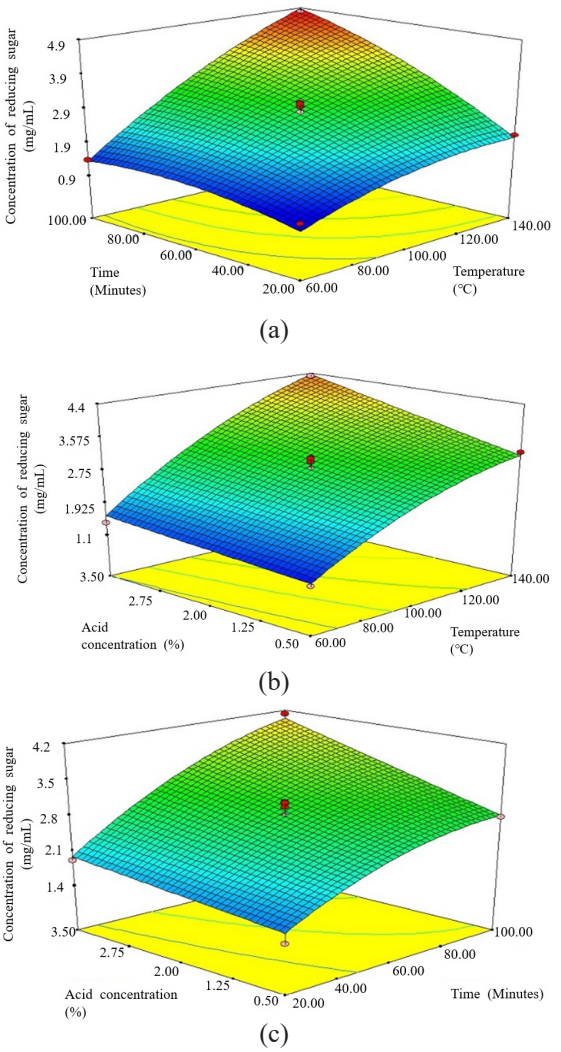


Figure 2: Contour plots of sugarcane bagasse representing the effects of pretreatment factors including (a) pretreatment time vs. pretreatment temperature, (b) acid concentration vs, pretreatment temperature and (c) acid concentration vs. pretreatment time on the concentration of reducing sugars (mg/mL).

sugar yield of sugarcane bagasse illustrates that, as the severity of pretreatment condition increases, the sugar yield could also be enhanced. As the pretreatment temperature, time, or acid concentration is increased, it can help in more disorganization of biomass structure. This provides more accessibility for enzymes towards their substrates. However, this trend could be observed until the pretreatment conditions reach a certain level, after that the trend showed a negative impact. This could be explained that when the temperature, time, or acid concentration were increased beyond a certain limit, it would lead to degradation of sugars [21], [47], [48].

In addition to this, contour plots representing the interaction between two factors at a time on the amount of reducing sugar production were also plotted (Figure 2). This plot could help to understand the effect of two factors on the response. For instance, Figure 2(a) showed that increasing pretreatment temperature and pretreatment time could increase the amount of reducing sugar similar to the single factor plot displayed in Figures 2(a) and (b). A similar trend was visible when the acid concentration and temperature were increased [Figure 2(b)]. The trend remained unaltered when the pretreatment was performed at the increased acid concentration for a prolonged time [Figure 2(c)].

At the highest temperature with extended pretreatment time, the yield of reducing sugar has also increased, which is represented as a red color zone in the contour plot [Figure 2(a)]. This contour plot was used as a tool for evaluating the optimum pretreatment conditions and observing their interacting effects.

3.2 *Biomass composition and optimized pretreatment conditions*

The biomass composition of the sugarcane bagasse was determined by Goering & Van Soest method [39]. The amount of cellulose, hemicellulose, and lignin was measured before and after pretreatment of the biomass, and results are tabulated in Table 3. As depicted in Table 3, the content of cellulose, hemicellulose, and lignin have decreased after the pretreatment of biomass at optimum conditions as given in Table 4. Acid pretreatment has previously been reported to decrease hemicellulose content in sugarcane bagasse [49]. This implies that the pretreatment has caused disintegration and also has enhanced the breakdown of the complex structure of sugarcane bagasse. This could have led to a decreased amount of cellulose, hemicellulose, and lignin after pretreatment.

Based on the RSM study, the mathematical model depicting the relations between reducing sugar concentration and (Y) and pretreatment factors (X_1, X_2, X_3) is given in Table 4. The RSM study predicts optimum pretreatment condition for pretreating sugarcane bagasse as the acid concentration of 3.5% at a

Table 3: Biomass composition of Sugarcane bagasse and Reducing sugar concentration released from enzymatic saccharification

Sample	Biomass Composition (%)			Reducing Sugar Concentration (g/L)
	Cellulose	*Hemicellulose*	*Lignin*	
Untreated biomass	48.717	23.277	20.716	1.44
Pretreated biomass*	32.494	1.666	11.464	4.41

*Pretreatment conditions: 3.5% H_2SO_4, 136.08°C, 75.36 min

Table 4: Predicted equation and predicted optimum pretreatment conditions for the production of reducing sugars obtained from sugarcane bagasse

Predicted model equation: **Reducing sugar concentration (mg/mL)** = –0.86616 + 0.037451 × Temp – 3.65380E-003 × time – 0.31926 x Conc. + 3.77076E-004 × Temp × time + 3.69853E-300 × Temp × Conc. + 3.55887E-300 × time × Conc. – 1.92255E-004 × $Temp^2$ – 1.74990E-004 × $time^2$		
Predicted optimum conditions of the highest reducing sugar concentration		
Temperature (°C)	**Time (mins)**	**H_2SO_4 Concentration (%)**
136.08	75.36	3.5
Predicted Sugar Concentration (mg/mL)	**Actual Sugar Concentration (mg/mL)**	**Difference (%)**
4.85	4.41	9.07

pretreatment temperature of 136.08°C for a pretreatment time of 75.36 min. At these optimum conditions, the model also predicts a yield of 4.85 mg/mL of reducing sugar concentration.

To validate the predicted model, pretreatment of sugarcane bagasse was repeated at the predicted optimum conditions and the reducing sugar concentration was calculated. It was noted that pretreatment at optimum conditions yields a reducing sugar concentration of 4.41 mg/mL, which was lesser than the predicted value of 4.85 mg/mL by 9.07%. However, in comparison with the untreated sugarcane bagasse, the reducing sugar concentration from pretreated sugarcane bagasse has increased by 3 folds. This could be attributed to the fact that acid pretreatment has hydrolyzed the complex structure of the biomass paving more accessibility for the enzyme to cellulose [49]. The enzyme can react with cellulose and release more sugar from pretreated biomass than untreated sugarcane bagasse. This result showcases the potential of sugarcane bagasse to be used as raw material for the biorefining process. The reducing sugar produced after pretreatment was used for fermentation with *S. cerevisiae* for ethanol production.

3.3 *Inhibitor analysis*

The process of pretreatment not only enhances the reducing sugar concentration but also can cause inhibitor formation as reported in previous studies [50]. Inhibitor analysis was carried out using liquid filtrate obtained after pretreatment of biomass and liquid hydrolysate obtained after enzymatic hydrolysate. These inhibitors were analyzed using GCMS and the results are shown in Table 5. Acetic acid, furfural, and 5-HMF are the main inhibitors identified in liquid filtrate after pretreatment, whereas acetic acid and furan methanol [51] are the main inhibitors identified in liquid hydrolysate obtained after enzymatic hydrolysis. Table 5 clearly shows an increase in acetic acid content in pretreated liquid filtrate when compared with the acetic acid content obtained from liquid hydrolysate of untreated biomass after enzymatic hydrolysis. This agrees with previous studies that pretreatment could release acetic acid as a byproduct from hemicellulose degradation [50]. The liquid filtrate also has furfural and 5-HMF present in it, which is released as a byproduct after xylose and glucose degradation respectively [52]. Furfural and 5-HMF are reported previously in many studies as common inhibitors produced after acid pretreatment [50]–[54]. These unwanted byproducts could interact with enzymes during enzymatic hydrolysis and can reduce enzyme efficiency, but this needs to be investigated further. Furthermore, the amount of acetic acid in liquid hydrolysate obtained after enzymatic hydrolysis has decreased and a new byproduct, furan methanol is also detected in it. The decreased acetic acid content could be the remnants after pretreatment

which were not removed post washing process. The formation of furan methanol can be explained as the reduction of furfural to a less toxic compound during fermentation [51], [55]. These inhibitor studies reveal the fact that the pretreatment of polysaccharides could generate fermentation inhibitors [56], [57].

Table 5: Inhibitor compounds identified from sugarcane bagasse during pretreatment and enzymatic saccharification

Sample	Inhibitor Compounds (mg/mL)			
	Acetic Acid	Furfural	5-HMF	Furanmethanol
Liquid hydrolysate of Control	0.957	n.d.	n.d.	n.d.
Liquid filtrate of pretreated Biomass	1.321	0.503	0.019	n.d.
Liquid hydrolysate of pretreated Biomass	1.109	n.d.	n.d.	0.034
Retention time (min)	10.763	13.415	19.619	13.568

n.d. = not detected

3.4 *FTIR analysis*

The chemical structure of pretreated and untreated sugarcane bagasse was studied by FTIR and the results were compared (Figure 3). In comparison, the spectra of untreated and acid pretreated biomass show differences. The peak exhibited between 3000 and 3500 cm^{-1} in both the samples represents the O-H stretching bands. The intensity of this peak has slightly increased after the acid pretreatment denoting a slight increase in the -OH or hydrogen bond in the pretreated biomass [58]. The peak at 1739 cm^{-1} could be attributed to the acetyl groups present in lignin or hemicellulose [49], [59]. The absence of this peak in the pretreated sample indicates the chance of removal of an acetyl group from hemicellulose or could be due to removal of hemicellulose after pretreatment. This could be correlated with the result of decreased hemicellulose content in Table 3 and the increased acetic acid content in Table 5. The peak observed in 1055 cm^{-1} could be a result of C-O stretching vibration between cellulose and hemicellulose [49] which is present only in the untreated sample. The peak exhibited by

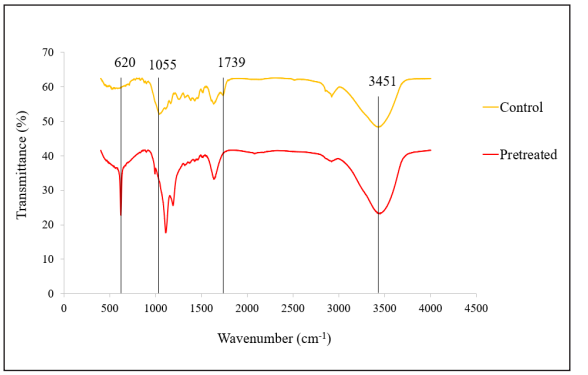


Figure 3: FTIR analysis of sugarcane bagasse.

the pretreated sample near 600 cm^{-1} could be due to the presence of lignosulfonates [60]. The FTIR studies represent that the pretreatment has caused changes in the chemical structure of sugarcane bagasse.

3.5 *Fermentation and ethanol yield*

Fermentation studies were carried out on both untreated and pretreated sugarcane bagasse. The ethanol yield from untreated sugarcane bagasse was 0.80 ± 0.013%, whereas pretreated sugarcane bagasse could produce only 0.50 ± 0.012% ethanol. This reduction in ethanol yield could be due to the presence of inhibitors in the pretreated sample as shown in section 3.3. Acetic acid and alcohol derivative of furfural was present in the hydrolysate as seen in Table 5. Acetic acid has been reported previously to have hindered the fermentation process and reduce ethanol yield [61]. In the presence of inhibitors, microorganism growth could be affected by the concentration of inhibitor or by the osmotic pressure [61]. This can lead to decreased ethanol yield in the pretreated sample when compared with the control. However, the yield of ethanol production obtained from the pretreated sample in this work was 145 g-ethanol/kg biomass, which was higher than other similar studies that used acid pretreatment on sugarcane bagasse (Table 6). This finding could be due to the higher concentration of sulfuric acid used in this work. Therefore, it could be suggested that even the efficiency of ethanol production was inhibited by inhibitor formation as a result of sulfuric acid pretreatment, and this optimized process still has an acceptable level of targeted products for further application.

Table 6: Previous reports on ethanol production from sugarcane bagasse

Substrate	Pretreatment Method	Pretreatment Conditions	Ethanol Yield	Reference
Sugarcane bagasse pith	Acid pretreatment	1% H_2SO_4, 121°C, 1.5 Bar pressure, 30 min	46.2 g ethanol/kg biomass after separate hydrolysis and fermentation of 18 h	[62]
Sugarcane bagasse pith	Acid pretreatment	1% H_2SO_4, 121°C, 1.5 Bar pressure, 30 min	66.4 g ethanol/kg biomass after simultaneous saccharification n and fermentation of 24 h	[62]
Sugarcane bagasse	Acid pretreatment	1% H_2SO_4, 121°C, 80 min	0.51 kg ethanol/ kg glucose	[63]
Sugarcane bagasse	Acid pretreatment	2% H_2SO_4, 155°C, 10 min	0.38g/g ethanol	[64]
Sugarcane bagasse	Acid pretreatment	0.5 % H_2SO_4, 140°C, 15 min	0.12g/g ethanol	[65]
Sugarcane bagasse	Acid pretreatment	1.25 % H_2SO_4, 140°C, 90 min	27.3 g ethanol yield from 160 g pretreated biomass	[66]
Sugarcane bagasse	Subcritical water hydrolysis	200°C, 15 MPa, Flow rate 5 mL/min, collection time of 19 min	2.20 ± 0.01 g/L ethanol	[67]
Sugarcane bagasse	Acid pretreatment	3.5% H_2SO_4, 136.08°C, 75.36 min	Concentration at 0.50 ± 0.012 %, 145 g-ethanol/kg biomass	This study

4 Conclusions

In this study, the pretreatment conditions for sulfuric acid pretreatment of sugarcane bagasse to produce maximum reducing sugar was optimized. RSM model was used to optimize the pretreatment conditions. The RSM study reveals that pretreatment with 3.5% H_2SO_4, at 136.08°C for 75.36 min can yield 4.41 mg/mL reducing sugar. Furthermore, pretreatment at optimum pretreatment conditions could produce 3.06 folds more reducing sugar than the untreated sugarcane bagasse. Even though, the pretreatment improves production of reducing sugar, it could also produce inhibitors, including acetic acid, furfural, and 5-HMF that could obstruct the fermentation process. Furthermore, the fermentation study could reveal that presence of inhibitors has reduced the ethanol yield to 0.50% in comparison with the untreated sugarcane bagasse. Further study needs to be done on pretreatment process to reduce the inhibitor effect on fermentation. Also, this study could provide insight into the usage of a mathematical model to optimize pretreatment conditions, along with the advanced idea to utilize the waste biomass to produce the value-added product.

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