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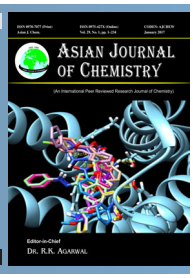

Production of Bioethanol from Cogon Grass (*Imperata cylindrical*)

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Imperata cylindrical or cogon grass is one of the abundant low-value by-products of agriculture waste in the world. The research had provided a discussion of how this non-valuable agriculture waste can be managed to become an alternative bioresource for bioethanol production. For effective bioethanol production, a pretreatment process is prerequisite to reduce the hemicellulose and lignin content in this recalcitrant lignocellulosic biomass. In this study, optimization of the temperature and concentration of acid and alkali were the major factor to ensure the effectiveness of the chemical pretreatment process. The pretreated conditions were optimized by pretreated *Imperata cylindrical* with H_2SO_4 and NaOH with 1.5, 2.0 and 2.5 % (w/v) at 90, 120 and 150 °C for 2 h. Among the test conditions, the optimal conditions of pretreatment were obtained in both 2 % (w/v) of sulphuric acid and sodium hydroxide at 90 and 150 °C respectively. The efficiency of both acid and alkaline pretreatment was evaluated through simultaneous saccharification and fermentation process. The highest ethanol yield was obtained with the *Imperata cylindrical* pretreated with sulphuric acid was 45.42 %.

Keywords: *Imperata cylindrical,* Lignocellulose, Bioethanol.

INTRODUCTION

Imperata cylindrical or cogon grass is an aggressive, perennial invader in the world. It can grow well in an infertile soil, which required only a minimal amount of water [1]. Its alleolopathic effects, reproduction rate and pyrogenic nature had allowed it to dominate vast tracts of degraded land and thus exclude other plant species. It is, therefore, considered as an ecological threat. Thus, the farmers preferred to burn them off [2]. This waste product is, however, can be fully utilized as it had been reported to have high cellulose and hemicellulose contents. It is, therefore, a sustainable resource for the bioethanol production. According to the research of indigenous weeds in six regions in the lower northern part of Thailand, *Imperata cylindrical* showed the highest ethanol yield of 548.4 ± 1.4 (L/ton) [3].

Conversion of lignocellulose materials to bioethanol in pretreatment process has been recognized as a crucial and rate-limiting step for the production of bioethanol. Pretreatment process secured a good yield of fermentable sugars and eliminated recalcitrant biomass [4]. This process involved the alteration of biomass that remove the lignin and disrupt the crystallinity of cellulose. Various type of chemicals, temperatures and pressures can use separately or in combination to pretreat and break down the structures of the lignocellulosic into their simple forms for further development or upgraded into a usable product for enhancing the enzymatic hydrolysis [5].

Alkaline pretreatment is an application that employed various alkaline reagents like sodium hydroxide, calcium hydroxide, potassium hydroxide and others which is basically known as the de-lignification process that solubilized significant amounts of hemicellulose. Saponification is one of the action mechanisms of alkaline pretreatment that lead to the cleavage of intermolecular ester bonds between the hemicellulose and lignin. Alkaline pretreatment basically depends on the reactivity between the alkali and material that produce swelling effect and fluctuation between internal surfaces of the cellulose which decreases the grade of crystallinity and disintegrated of the lignin-carbohydrate-lignin linkage [4,6].

Acid pretreatment is the simplest and faster method used to obtain amorphous hemicellulosic and cellulosic fraction through the disruption of glycosidic bond and cleavage of acetyl ester groups. Acid has the capability to reduce the temperature and the retention time of the processing system and disturb the hydrogen bond between cellulose and forms homogeneous gelatine in lignocellulosic materials. Dilution of cellulose with water at modest temperatures and retention time provide rapid hydrolysis to monosaccharides [7]. Temperature, concentration of acid/alkali, time and solid-to-liquid ratio are the parameters that played a significant role in obtaining optimum

sugar recovery and minimize the production of inhibitors during pretreatment process [8].

In this study, the main objectives of this research were to determine the effect of reaction for the operating parameter such as the temperature and concentration of acid and alkaline solution with the performance of the pretreatment process. The effectiveness of acid and alkali pretreament on *Imperata cylindrical* was also investigated and evaluated. The yielding of bioethanol was also determined through the performance in simultaneous saccharification and fermentation process.

EXPERIMENTAL

Biomass preparation: *Imperata cylindrical* or cogon grass was obtained from the forest area at Jeli, Kelantan. The sample was then dried at 65 °C about 3 days to ensure the sample collected was completely dried. After that, the sample was blended to the size less than 3 mm. The prepared material was then stored in plastic bags, sealed at room temperature.

Pretreatment: A factorial experimental design was conducted to identify the effect of pretreatment condition, *i.e.* acid and alkali concentration and the reaction temperature on the reducing sugar yield. Sulphuric acid and sodium hydroxide concentration of 1.5, 2.0 and 2.5 % w/v were examined at 90, 120 and 150 °C for 2 h with a solid liquid ratio of 1:10. The hydrolyzate liquors obtained was then centrifuged at 2500 rpm for 5 min where three 100 µL samples were taken in order to determine the contents of reducing sugar [4,9] by 3,5-dinitrosalicylic acid solution (DNS) method with glucose as standard [10].

Reducing sugar determination using dinitrosalicyclic acid method: A standard curve for preparation of reducing sugar was prepared using different concentration of glucose solution (0-1000 µg/mL) in distilled water. The test was carried up with 0.5 mL of DNS reagent, which added to 0.5 mL of each concentration of glucose in the capped test tubes. The mixture was then boiled for 15 min to develop the colour. After that, 1mL of sodium potassium tartarate was added to stabilize the colour of the mixture. The mixture is then added with 3 mL of distilled water after heated for 5 min. After cooling at room temperature, the absorbance reading was recorded spectrophotometrically at 540 nm.

Preparation of yeast peptone dextrose (YPD): Ingredients contained (g/L) yeast extracts 10, peptone 20 and D-glucose 20 were weighed and poured into the media bottle. The total volume has made to reach 100 mL in media bottle with distilled water. The media was then shaken to ensure the solution mixed well and the pH was adjusted to 5. The solution was sterilized using autoclave at 121 °C for 15 min before stored in freezer at 4 °C.

Preparation of *Saccharomyces cerevisiae* inoculum: The yeast cells, *Saccharomyces cerevisiae* was grown by inoculating baker's yeast in yeast peptone dextrose agar that contained (g/L) yeast extract 10, peptone 20, D-glucose 20 and agar-agar powder 20 and incubated at 37 °C for 2 days. A loop of yeast inocula are picked and grown in conical flasks that contain yeast peptone dextrose medium. The flasks are then incubated in an orbital shaker at 150 rpm, 37 °C for 24 h.

Simultaneous saccharification and fermentation (SSF): Simultaneous saccharification and fermentation was carried out but adding autoclave substrate (100 g), to the bioreactor which consists of 500 mL basal medium, yeast inoculum and commercial enzyme. The steps were then proceed with the bioreactor vessel set up and the pH probe was calibrated by gathering two reference buffer which are NaOH and HCl in reagent bottles at pH 5. The bioreactor was then connected to a laptop to control all the probes, which were set up and the fermentation process will be carried out for 24 h.

Statistical analysis: The optimization of the pretreatment conditions was investigated using an effective experimental design procedure, SPSS 22.0 to perform analysis of variance. Two-way ANOVA was used to identify the significant effects from each factor as well as the interaction between the factors. The data obtained was adjusted using Tukey's adjustment and the significant differences were analyzed to compare the pretreatment parameters.

Analytical method: Ethanol concentration was analyzed using high performance liquid chromatography (HPLC) system to quantify the ethanol yield. Samples were prepared from fermentation broth and filtered using 2 µm syringe filter. This column was run at 40 °C with a flow rate of 1.0 mL/min using 0.05 M *meta*-phosphoric acid diluted with water as the mobile phase. Sample injections were 20 µL and the run time was 40 min.

RESULTS AND DISCUSSION

Glucose calibration: A calibration curve was drawn to determine the concentration of glucose from cogon grass. A standard calibration curve was prepared by using 0-1000 µg/mL concentration prepared in distilled water. From the graph, the obtained curve regression equation in y = 0.0006x with correlation (R^2) = 0.9926. Table-1 showed the data used to plot the glucose calibration curve.

TABLE-1
RESULTS OF MEASUREMENT OF GLUCOSE CONCENTRATION FOR CALIBRATION CURVE

Glucose concentration (µg/mL)	Absorbance at 540 nm
0	0.000
200	0.080
400	0.205
600	0.339
800	0.462
1000	0.581

Determination of reducing sugar from *Imperata cylindrical* was accomplished using 3,5-dintrosalicyclic acid method. 3,5-Dintrosalicyclic acid test was used to determine the presence of the free carbonyl group (C=O) in the reducing sugar. The basic principle of 3,5-dintrosalicyclic acid method is through the oxidation of aldehyde group to carboxyl group and simultaneous reduction of 3,5-dinitrosalicyclic acid to 3-amino, 5-nitrosalicyclic in the sugar structure. The presence of 3-amino, 5-nitrosalicyclic, an aromatic compound with maximum absorption at 540 nm allowed a quantitative spectrophotometer measurement of the amount of RS present. The amount of RS is proportional to the depth of colour intensity when the sugar

concentration of exceed certain range where different sugars will yield different colour intensity [9].

Besides that, 3,5-dintrosalicyclic acid reagent composed of compounds such as dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulphite and sodium hydroxide that are indispensable for the development of proper reaction. Sodium hydroxide acts as a stabilizer which provides a redox reaction between the reducing sugars and 3,5-dintrosalicyclic acid. Sodium potassium tartrate presents increase the ion concentration and, therefore, decrease the oxygen concentration in the solution while phenol is used to increase and enhance the colour density produced. Sodium sulphide is used to absorb dissolved oxygen that may potentially interfere with reducing sugar oxidation [11,12].

Optimization of pretreatment condition of cogon grass with H_2SO_4: Cogon grass which have been pretreated with 1.5, 2.0 and 2.5 % w/v H_2SO_4 at 90, 120 and 150 °C for 2 h were investigated. The results were shown in Fig. 1. From the results, it can be seen that the optimum H_2SO_4 concentration of 2 % and temperature of 90 °C was found to attain highest absorbance reading. The results from this study showed low absorbance reading of H_2SO_4 for almost every condition, suggested that hydrolysis process is prone toward degradation with the increased of acid concentration. This is because a high concentration of acid might result in the degradation of reducing sugar to form other by-products, which may lead to a low sugar recovery [10].

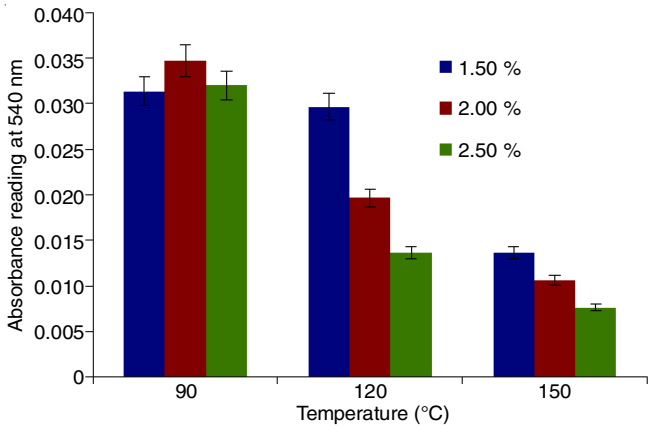

Fig. 1. Absorbance reading obtained from cogon grass that pretreated with 1.5, 2.0 and 2.5 % (w/v) sulphuric acid at 90, 120 and 150 °C

A soft pretreatment condition may lead to the formation of the sugar-rich product. A harsh hydrolysis condition however, may cause the sugar recovery process in the liquor decreases. Besides that, a high temperature at the elevated acid concentration had produced a decreasing in the overall sugar yield. This may be due to the degradation process, which occurs causing the cellulose recovery in the pretreated sample decreases [13]. At moderate temperature, the dilute sulphuric acid effectively hydrolyzed hemicellulose and recovers it as dissolved sugar [14].

Fractional factorial design of H_2SO_4 pretreatment: The optimizations of pretreatment conditions had been an important problem in the development of the economical scheme bioprocesses. Two-way analysis of variance (ANOVA) was conducted to evaluate the significant influences of the effects of temperature and concentration of acid on the reducing sugar yields. This indicated that the highest the absorbance reading, the highest the reducing sugar concentration (Table-2). The independent variables were the temperature and the concentration of acid while the dependent variable was the absorbance reading. Table-3 shows the statistical analysis of the effects of the temperature and concentration of acid on the absorbance reading. Both temperature and concentration of H_2SO_4 had shown statistically significant effects (Sig = 0.000, $P < 0.05$) on the reducing sugar formation. The model was highly significant with ($p < 0.01$) and have $R^2 = 0.988$. The ANOVA output for absorbance reading had confirms the significance of the factors as well as each interaction terms and showed that the temperature and concentration of sulphuric acid offer the greatest influenced on the reducing sugar yield. Fig. 1 showed the reducing sugar loss under certain pretreatment conditions. This loss may probably due to the degradation of sugars into furfural as a result of harsh pretreatment conditions.

TABLE-2
ABSORBANCE READING OBTAINED FROM COGON GRASS THAT PRETREATED WITH 1.5, 2.0 AND 2.5 % (w/v) SULPHURIC ACID AT 90, 120 AND 150 °C

Temperature (°C)	Concentration of H_2SO_4 (% w/v)		
	1.5	2.0	2.5
90	0.0313	0.0346	0.0320
120	0.0297	0.0197	0.0137
150	0.0137	0.0107	0.0077

Optimization of pretreatment condition of cogon grass with NaOH: Cogon grass was pretreated with 1.5, 2.0 and 2.5 % w/v NaOH at 90, 120 and 150 °C for 2 h were investigated. The results of the alkali pretreatment in Fig. 2 showed that the optimum NaOH concentration and temperature for

TABLE-3
STATISTICAL ANALYSIS OF THE EFFECTS OF TEMPERATURE AND CONCENTRATION OF H_2SO_4 ON THE ABSORBANCE READING

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	0.003[a]	8	0.000	189.957	0.000
Intercept	0.012	1	0.012	7132.787	0.000
Temperature	0.002	2	0.001	626.362	0.000
Concentration	0.000	2	0.000	65.553	0.000
Temperature × Concentration	0.000	4	5.911×10^{-5}	33.957	0.000
Error	3.133×10^{-5}	18	1.741×10^{-6}		
Total	0.015	27			
Corrected total	0.003	26			

[a]$R^2 = 0.988$ (Adjusted $R^2 = 0.983$)

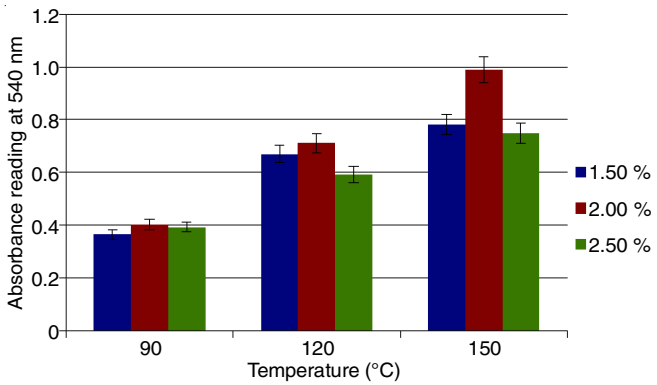

Fig. 2. Absorbance reading obtained from cogon grass that pretreated with 1.5, 2.0 and 2.5 % (w/v) sodium hydroxide at 90, 120 and 150 °C

pretreatment of cogon grass was 2 % w/v NaOH at 150 °C. Table-4 showed that the 2 % w/v NaOH caused a higher absorbance reading compared to other concentrations of NaOH. The increasing NaOH loading to 2.5 % w/v is, however, decreasing the absorbance reading. This is because, when the concentration of NaOH exceeded a certain limit, there will be a substantial loss of carbohydrate during the pretreatment process.

TABLE-4
ABSORBANCE READING OBTAINED FROM COGON GRASS THAT PRETREATED WITH 1.5, .0 AND 2.5 % (w/v) SODIUM HYDROXIDE AT 90, 120 AND 150 °C

Temperature (°C)	Concentration of NaOH (% w/v)		
	1.5	2.0	2.5
90	0.3657	0.4023	0.3937
120	0.6703	0.7117	0.5920
150	0.7810	0.9880	0.7500

The study also indicated the optimal temperature for 2 % w/v NaOH pretreatment is at 150 °C. An increase in temperature maximises and accelerates the higher delignification and swelling structure in cogon grass, which then caused the pretreated cogon grass easier to hydrolyze by enzyme cellulase. However, high severity pretreatment condition may also lead to the undesirable sugar loss [15]. The mechanism in alkaline pretreatment is believed to be disrupting the cross-linking bond between cellulose, hemicellulose and lignin, which leads to high susceptibility of cellulose component for saccharification process.

Fractional factorial design of NaOH pretreatment: Statistical analysis of pretreatment data, which examines the relationship between effects of temperature and concentration of NaOH on the absorbance reading is summarized in Table-5 which have R^2 values of 0.985. The data from two-way analysis of variance (ANOVA) also showing high statistically significant effects on reducing sugar formation. The model was highly significant and correlation with ($p < 0.01$) and have $R^2 = 0.985$. The ANOVA output for absorbance reading had confirms the significance of the factors as well as each interaction terms and showed that the temperature and concentration of sodium hydroxide offer the greatest influenced on the reducing sugar yield. Fig. 2 showed that the absorbance reading obtained after pretreatment process for each sodium hydroxide concentration. It showed that the increasing of temperature or sodium hydroxide concentration resulted in the increasing of reducing sugar concentration. Low alkaline concentration (< 4 % w/w) is, however, are mostly suitable at high temperature. These pretreatment conditions loosen and break down the biomass structure, increasing the internal surface area, which enhanced glucose yields [16].

Ethanol calibration: An ethanol calibration curve was drawn to determine the concentration of ethanol yield from cogon grass. A standard calibration curve was prepared by using absolute ethanol at different concentrations. From Table-6, the obtained curve (Fig. 3) regression equation was obtained y = 352.27 with correlation (R^2) = 0.949.

TABLE-6
RESULTS OF MEASUREMENT OF ETHANOL CONCENTRATION FOR CALIBRATION CURVE

Ethanol concentration (%)	Retention time (min)	Peak area (a.u)
0	0	0
25	4.919	2765
50	4.514	14519
75	4.457	27544
100	4.296	37442

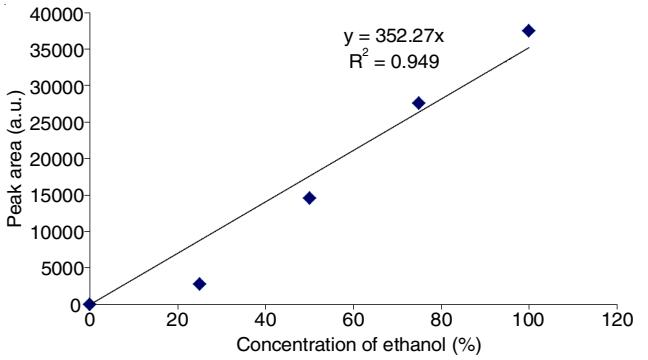

Fig. 3. Ethanol calibration graph between the peak area and ethanol concentration

TABLE-5
STATISTICAL ANALYSIS OF THE EFFECTS OF TEMPERATURE AND CONCENTRATION OF NaOH ON THE REDUCING SUGAR YIELDS

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	1.058[a]	8	0.132	114.375	0.000
Intercept	10.658	1	10.658	9218.608	0.000
Temperature	0.933	2	0.467	403.520	0.000
Concentration	0.074	2	0.037	32.008	0.000
Temperature × Concentration	0.051	4	0.013	10.986	0.000
Error	0.021	18	0.001		
Total	11.737	27			
Corrected total	1.079	26			

[a]$R^2 = 0.981$ (Adjusted $R^2 = 0.972$)

Ethanol production by simultaneous saccharification and fermentation: The pretreated samples were then subjected to simultaneous saccharification process by *Saccharomyces cerevisiae* at 37 °C. It is then hydrolyzed enzymatically by using commercial enzyme from *Aspergillus niger*. In order to verify the best chemical pretreatment, simultaneous saccharification and fermentation process was carried out. A higher ethanol yield was obtained when using sulphuric acid pretreatment in the amount of 45.42 % (Table-7). This shows that sulphuric acid fractionates celluloses components and remove hemicellulose better than sodium hydroxide. Dilute acid pretreatment was reported to be effective in increase cellulose crystallinity and disrupting lignin component. It is considered as the most significant sugar depolymerization method [17].

TABLE-7
ETHANOL YIELD (%) THAT TREATED WITH 2.0 % w/v SULPHURIC ACID AT 90 °C AND 2.0 % w/v SODIUM HYDROXIDE AT 150 °C

Type of pretreatment	Peak area	Ethanol yield (%)
Sulphuric acid	16000.10	45.42
Sodium hydroxide	5710.30	16.21

Inhibitory compounds identification: Under a harsh condition reducing sugar is, however, were not be completely used at the end of the fermentation process. This might be due to the presence of high ethanol concentration, insufficiency of nutrient in the fermentation broth [18] and present of the inhibiting compounds. In this study, inhibitory compounds in cogon grass were identified through GC-MS methods. The identified inhibitors are showed in Tables 8 and 9.

TABLE-8
INHIBITING COMPOUNDS IDENTIFICATION FROM FERMENTATION BROTH (ACID PRETREATMENT)

Compounds	
Propanoic acid, 2-oxo-,methyl ester	$C_4H_6O_3$
Furfural	OC_4H_3CHO
2-Furan methanol	$C_5H_6O_2$
Pentyl ester of butanoic acid	$C_9H_{18}O_2$

TABLE-9
INHIBITING COMPOUNDS IDENTIFICATION FROM FERMENTATION BROTH (ALKALINE PRETREATMENT)

Compounds	
Butanedioic acid, 2,3-bis(acetyloxy)-	$C_8H_{10}O_8$
Furan methanol	$C_5H_6O_2$
Phenol, 2-methyl-5-(1-methylethyl)-	$C_4H_6O_3$

On the top of that, an efficient and rapid fermentation of the lignocellulosic hydrolyzates is limited because in addition to simplest sugars, a range of the inhibiting compounds is generated during the pretreatment process. Inhibitory compounds are mostly formed when some of the carbohydrates were degraded. Besides, inhibitory compounds were divided into three categories, the furans, weak acids and phenolic compounds [19]. Phenolic and aromatic compounds are formed from lignin during pretreatment of lignocellulosic biomass or regardless of whether an acid catalyst is added. Carboxylic acids were formed in an acidic environment. Furan aldehyde such as furfural was formed through dehydration of pentose and hexoses sugar [20]. The effect of these inhibitory compounds may inhibit the growth of *Saccharomyces cerevisiae* and the product yield.

Conclusion

As a conclusion, the production of biofuel from renewable resources had becoming an important issues encounter in sustainable development in human society. Cogon grass contains various carbohydrates compounds was an interesting choice for bioethanol production by using *S. cereviciae*. In this study, both acid and alkaline pretreatment of cogon grass was highly effective in enzymatic hydrolysis and maximizing ethanol yield. The optimal pretreatment conditions were achieved in both 2 % (w/v) of sulphuric acid and sodium hydroxide at 90 and 150 °C, respectively. Besides, the efficiency of both acid and alkaline pretreatment was evaluated through simultaneous saccharification and fermentation process where the highest ethanol yield obtained was 45.42 % by the cogon grass pretreated with 2 % (w/v) sulphuric acid at 90 °C.

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