OPTIMIZED PRODUCTION OF BIOETHANOL BY FERMENTATION OF ACID HYDROLYZED-CORN STOVER EMPLOYING *SACCHAROMYCES CEREVISIAE* YEAST STRAIN

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ABSTRACT

In this study, corn stover was converted into ethanol using a locally-fabricated bioreactor and process conditions were optimized. The corn stover biomass used as substrate was milled, screened to 200 µm and hydrolyzed with between 0.1-0.5 M HCl. The hydrolysis experiment was carried out for substrate concentrations of 20, 25, and 30% (w/v) of milled bagasse prepared in a 1000 mL glass jar containing distilled water. For each substrate concentration, the time, temperature, and acid concentration were varied between 10 – 60 min., 80 – 97 °C, and 0.1 – 0.5 M, respectively to find the optimum glucose yield. Glucose concentration in the optimum hydrolysate sample was determined using glucose oxidase method. Fermentation experiment was conducted in the bioreactor using 700 ml of the hydrolysate and Saccharomyces cerevisiae supplemented with minerals to yield ethanol of 21.47 g/L after 48 hours. A linear regression model developed after analysis of variance was able to predict the concentration of glucose produced during the acid hydrolysis, and the optimum ethanol yield of 21.47 g/L compares well with previous reported yield values found in literature.

Keywords: Hydrolysis, Glucose, Fermentation, Lignocellulose, *Saccharomyces cerevisiae*.

1.0 Introduction

Production of biofuel has shifted towards the use of farm field wastes as feedstock. This is associated with the quest to search for alternative fuel (energy) to replace fossils and with the environmental advantages of ethanol. To secure a sustainable energy supply there should be efficient conversion of lignocellulose into renewable chemicals and fuel (Elmore *et al*., 2020).

Lignocellulose which is also known as plant dry material is the most abundant raw material used for the production of bioethanol (Sun and Cheng, 2002) and a great prospect in biorefinery (FitzPatrick *et al*., 2010). It is the structural component of all plant matter that has high energy value. It cannot be used directly for human consumption. It holds great promise for renewable energy production (Hahn-Hägerdal *et al.,* 2006). To meet energy required, bioethanol has to be produced on a large scale (Karagoz *et al.* ,2019). Large-scale cellulosic ethanol production employs the use of materials such as agricultural residues, forest woody feedstock, energy crops, and municipal solid waste via anaerobic digestion. Also, the production of bioethanol on a large scale does not in any way compete for food with human directly or indirectly (Liu *et al*., 2019).

Ethanol can also be used in the production of germicide, antifreeze and intermediate for other organic materials. Interest in the production of biofuel from agriculture wastes is driven by several reasons such as: global search for alternative source of energy and transportation fuel to replace the depleting fossil fuel (Ragauskas *et al.,* 2006). Bioethanol is a natural product and is manufactures by the fermentation of plants containing sugar and starch. Ethanol is pollution free, biodegradable, renewable, cause no climate change and the by-product of fermentation can be used as animal feedstock.

A variety of feedstock can be used for bioethanol production. However, corn residues have been posited as the feedstock which has the highest potential for bioethanol production (Kim and Dale 2004). All part of corn plant has different potentials (Li *et al*. 2020), Corn stover refers to stalks, leaves and cobs that remain on the fields after the corn was harvested. This biomass can be used in producing ethanol. Corn stover is the primary biomass source being used for producing cellulosic ethanol in the United States (Wilhelm *et al.* 2007).

Stover has the advantage of not being a food source like corn itself, and as a by-product of corn production, it has lower production costs. Kadam and McMillan (2003) have identified corn stover as a sustainable feedstock for ethanol production. Estimates of corn availability has been widely discussed in the literature. Abbasi and Abbasi (2010) gave an estimate availability of corn stover to around 200 million dry tones/ yr with about 20-60 % which can be harvested sustainably. The study of Correll (2014) reported that 58 % of the corn stover can be harvested on the sustainable basis. Harvesting of the corn stover in excess brings about challenges to the growers. The biofuel producer may harvest excess stover which may capture additional value of the crop. Higher corn has brought about more increase in corn

The present study focuses on production of ethanol using corn stover and *saccharomyces cerevisae.* The corn stover which is the substance used was pre-treated before used for the production, and the process was optimized to improve the ethanol yield from a fabricated bioreactor.

2.0 MATERIALS AND METHODS

2.1 Materials

A locally fabricated bioreactor, water bath, weighing balance, test tubes, conical flask, beaker, glass stirrer, petri dish, and UV-Spectrophotometer (NIR Model). Also, the analytical grade chemicals and reagents used were distilled water, yeast extract agar, Sodium hydroxide, Ammonium Sulphate, Potassium hydrogen phosphate, Magnesium Sulphateheptahydrate, Cell biomass, concentrated ethanol, Hydrochloric acid, Standard glucose, Phenol, Sodium acetate, Glacial acetic acid, Fermocozme, Peroxidase, and 4-aminophenazone. Corn Stover obtained from Oko-Oba, Tanke – Ilorin.

2.2 Experimentation

2.2.1 Preparation of substrate

The Corn Stover was air-dried, milled and screened to 2*mm* particles to increase its surface area and make the cellulose susceptible for hydrolysis. It was then stored at room temperature for subsequent use.

2.2.2 Preparation of reagents

Both the Phenol and Enzyme reagents were prepared as follows: About 2.0 g phenol and 9.0 g NaCl were dissolved in one liter of distilled water to make phenol solution, and also 12.88 g sodium acetate, 0.32 cm^3 glacial acetic acid, 15.0 cm^3 fermocozyme, 15 mg peroxidase and 301 mg 4-Aminophenazone were dissolved in distilled water and make up to one liter. The solution were stored in the refrigerator at 4 ^{0}C. Three test sets of tubes were used with the wavelength 510 nm and temperature 37 ^{0}C and a blank was prepared with distilled water to set the equipment to Zero calibration.

2.2.3 Acid hydrolysis of corn stover

In the *HCl* acid hydrolysis experiment, three different substrates with concentrations of 20, 25 and 30% (w/v) were prepared by the addition of 20, 25 and 30 g of Corn Stover to 200 ml dilute *HCl* (50:50 vol.% water to acid) respectively. For each case of 20 and 30 % substrate concentration, the effects of hydrolysis time (10 – 60 min), temperature (80 – 97°C), and acid concentration (0.1 – 0.5M) were investigated by varying these parameters in 2^3 Factorial experiment (8 runs). Samples were withdrawn regularly and cooled before filtering for 15 minutes after stopping the hydrolysis by neutralizing the sample with 0.1M *NaOH*. The filtrates were immediately kept in a fridge for glucose analysis. The glucose concentrations measured in each of the 8 experimental runs were measured as the response, and the optimum condition with the highest measured glucose concentration was identified.

2.2.4 Measurement of glucose concentration

The glucose analysis was carried out using glucose oxidase method. In this case, Phenol reagent was prepared by dissolving 2.0 g phenol and 9.0 g *NaCl* in 1 *L* distilled water. Enzyme reagent also was prepared by measuring and dissolving in water, 12.88 g sodium acetate, 0.32 cm^3 glacial acetic acid, 15.0 cm^3 Fermocozyme, 15 mg peroxidase, and 301 mg 4-Aminophenazone, and making up to 1 liter. These were stored in the refrigerator at 4 °C.

2.3 Fermentation Experiment

2.3.1 Pre-culture medium

Saccharomyces cerevisiae (Baker's yeast) was gotten from the Department of Microbiology, University of Ilorin and maintained on agar at room temperature.

2.3.2 Preparation of the subculture

200 ml of distilled water was measured into a conical flask, 4.6 g of yeast extract agar was added into the conical flask and stirred until a homogenous mixture was obtained. The solution was then autoclaved at 121 ^{0}C and 15 psi for 15 min. After autoclaving the solution, it was poured into a petri dish and then allowed to cool. Cells of saccharomyces cerevisiae was transferred into the petri dish aseptically in a sterile condition and incubated at 30 ^{0}C for 6 hours.

2.3.3 Sterilization of fermenter

The bioreactor was sterilized using 70 % concentrated ethanol to reduce the contaminant in the reactor at 90 ^{0}C for 20 min with sterile distilled water.

2.3.4 Preparation of fermentation media

About 700 ml of the hydrolysate at the optimum glucose yield which correspond to 20 % substrate concentration during hydrolysis was

measured into a conical flask, 2.8 g of yeast extract, 1.4 g of ammonium sulphate, 1.4 g of potassium hydrogen phosphate, 0.525 g of magnesium sulphateheptahydrate were also measured into the conical flask and the pH was adjusted to 5.5 using NaOH. The fermentation media was autoclaved at 121 ^{0}C, 15 psi for 15 minutes to maintain sterile condition.

2.3.5 Bio-ethanol production from corn stover

Pre-culture media of micro-organism was prepared with a potato dextrose agar PDA which is mostly used for growing bacteria and fungi. The agar was transferred into a subculture media containing similar material as the fermentation media. De-ionized water was poured into the bioreactor vessel after which the bioreactor was plug to power supply and the temperature is set to 100 °C for 30 min. The bioreactor was allowed to cool to room temperature and the fermentation medium was poured into the bioreactor vessel, the subculture medium was also inoculated into the bioreactor. It was then plugged back to power supply. The temperature, pH and agitation speed were set at 34 ℃, 5.5 and 150 rpm respectively. Fermentation was allowed to continue for 48 h while samples were taken at 4 h intervals samples were analyzed using the spectrophotometer at a wavelength of 600 nm.

2.3.6 Estimation of ethanol content

Ethanol in fermented sample was estimated by the method of Caputi *et al.* (1968).

2..2.7 Standard stock of ethanol

Ethanol standards were made by using ethanol-water solution in the range of 0-20 ethanol (g/l).

2.2.8 Preparation of potassium dichromate solution

Potassium dichromate solution was prepared by adding 325 ml conc. H_2SO_4 to 400 ml distilled water in 1-liter volumetric flask. After mixing and cooling (80- 90 ˚C), 33.768 g $K_2Cr_2O_7$ was added and then final volume of 1 liter was made with distilled water at 20˚C.

2.2.9 Preparation of standard curve

The standard curve was prepared by taking 1 ml of each concentration of the standard solution [0-20 (g/l)] in a 100 ml volumetric flask containing 25 ml of potassium dichromate solution. The samples were heated at 60 ˚C for 20 min in a water bath and then cooled and diluted to 50 ml with distilled water. Absorbance was recorded at a wavelength of 600 nm using SPCTRONIC GENESYS Spectrophotometer

2.2.10 Estimation of ethanol in the sample

About 1 ml of alcoholic sample was added directly to the distillation flask, diluted to 30 ml with distilled water and then distilled. Distillation was carried out at 70-72 ˚C and 20 ml of distillate was collected in a 50 ml volumetric flask containing 25 ml of potassium dichromate solution. The contents in the volumetric flask were heated at 60 ˚C in a water bath for 20 min and final volume was made to 50 ml with distilled water. After mixing and cooling the contents of the flask, the absorbance was recorded at 600 nm. The amount of ethanol in each sample was determined by using the standard curve of ethanol.

3.0 RESULTS AND DISCUSSION

3.1 Acid Hydrolysis Corn Stover Slurries

The summary of the experimental and predicted values for the production of glucose for each of the slurries concentrations of 20, 25 and 30 % corn stover given in Table 1. The corresponding analysis of variance (ANOVA) is presented in Table 2 for the slurry that give the best yield. The F value is the ratio of the mean square due to regression to the mean square due to the real error. The response taken from Table 2 reveals that the linear term of time (x_1), temperature (x_2) and acid concentration (x_3) have remarkable effects on the glucose concentration. The significance of each coefficient was determined using p-value ($p < 0.05$) and the smallest p-value indicates high significance of the corresponding coefficient. It can be seen that the variables with the largest effect was temperature. All the linear terms are significant. The highest glucose concentration was achieved at time of 60 min; temperature 97 ^{o}C and acid concentration 0.1 M. The modeling equation is given by Equation 1;

$$Y_n = 1.14 + 0.046\, x_1 + 0.069\, x_2 - 0.042\, x_3 + 0.016\, x_1x_2 - 0.035\, x_1x_3 + 0.048\, x_2x_3 \quad (1)$$

Table 1: Summary of Factorial Experimental Design Matrix for Corn Stover Slurry Acid hydrolysis

Slurry	Time(min)	Temp. (˚C)	Acid conc. (M)	Glucose conc. (g/l)	Predicted value(g/l)
20 %	60	97	0.1	1.29714	1.30
25 %	60	97	0.1	0.98972	0.99
30 %	10	80	0.1	1.17879	1.18

Table 2: Regression Analysis (ANOVA) for the Acid Hydrolysis of 20 % Corn Stover Slurry

Factor	SS	DF	MS	F	P-value
Model	0.10	6	0.017	4089.00	0.0120
x_1-*Time*	0.017	1	0.017	4225.00	0.0098

x_2-Temperature	0.038	1	0.038	9409.00	0.0066
x_3-Acid Conc	0.014	1	0.014	3481.00	0.0108
x_1x_2	2.159E-003	1	2.159E-003	529.00	0.0277
x_1x_3	9.800E-003	1	9.800E-003	2401.00	0.0130
x_2x_3	0.018	1	0.018	4489.00	0.0095
Residual	4.082E-006	1	4.082E-006		
Cor Total	0.10	7			

SS: sum of squares; DF: Degree of freedom; MS: square means.

Where Y_n is the response, i.e. glucose concentration (g/l) and x_1, x_2 and x_2 are the coded values of test variables: time, temperature and acid concentration respectively.

Figure 1 shows the interaction between the predicted and actual value. R- coefficient of correlation = 0.978; R^2- coefficient of determination = 0.956; Adjusted R^2 = 0.9997. The fit of the model is expressed by the value of R^2 which was found to be close to 1.00. This shows that Equation 1 is a suitable model to describe the response of the experiment.

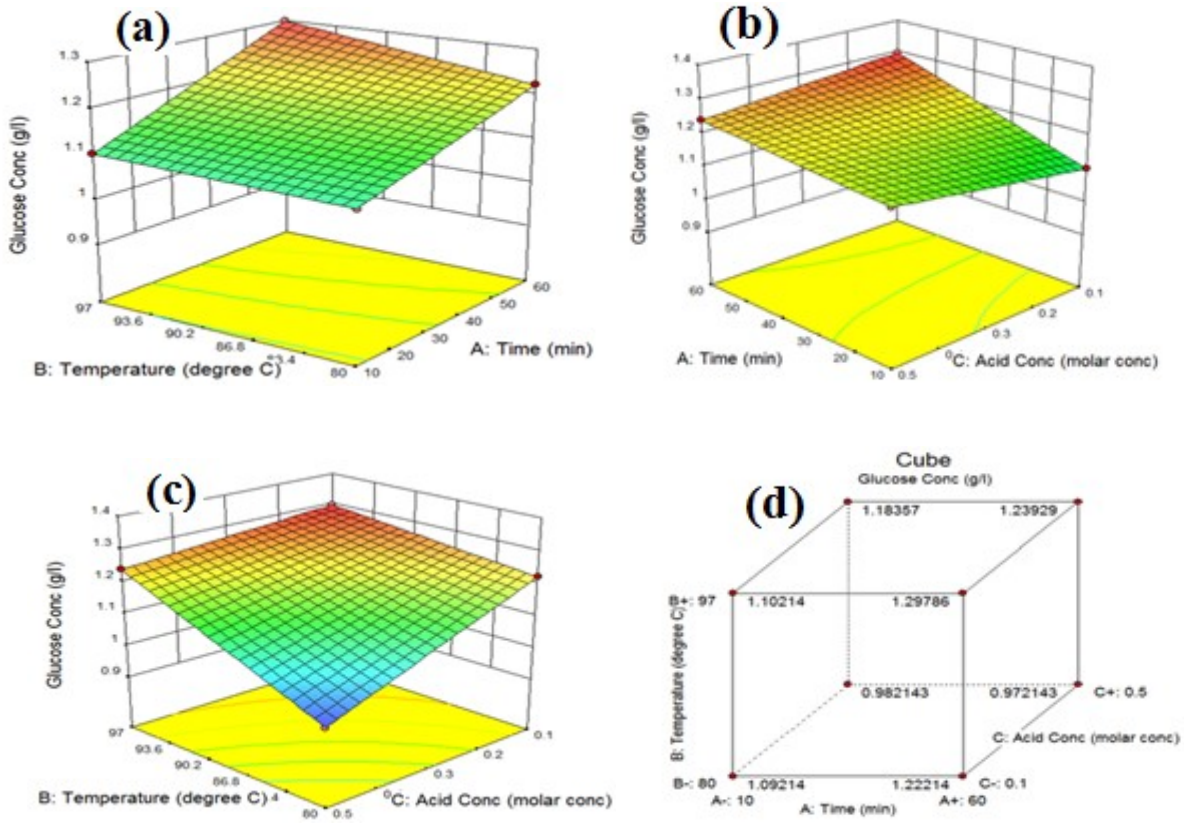

Figure 1: Interaction among Variables on the Glucose Yield Using 20 % of Corn Stover Slurry

3.1.1 Effects of interaction between two variables on the glucose yield

The effect of the interaction between two variables on the glucose concentration is presented in Figure 1. Figure 1(a) shows the interaction between the effect of temperature and time on glucose concentration yield while the acid concentration is kept constant at 0.1 **M**. Figure 1(b) shows the interaction effect of time and acid concentration on glucose concentration yield while the temperature is kept constant at 97 ˚C. Figure 1(c) shows the interaction effect of temperature and acid concentration on glucose concentration yield while the time is kept constant at 60 min. Figure 1(d) shows the interaction between the three factors. In Figure 1(a-c) it was observed that the glucose concentration yield increases with increasing operating variables. The optimal values of time, temperature and acid concentration was

estimated in actual units were 60 min, 97 ºC, 0.1 **M**, respectively with predicted reducing sugar yield of 1.29714 g/l. The result from the analysis above was used to optimize the glucose concentration yield. Figure 1(d) shows the ramps and the desirability of the model at maximum time and temperature but minimum acid concentration to give a maximized glucose concentration yield with a desirability of 1.000.

3.4 Glucose and Ethanol Contents

Table 1 shows optimal glucose yield of 1.297 g/l at 0.1 **M** acid concentration, temperature 97 ^{0}C and time 60 min. The results as presented in Table 3 shows the corresponding ethanol yield of 21.47 g/L and the literature comparison of the present study. You *et al.,* (2017) reported ethanol concentration (68.24 g/L), from sugar cane bagasse, Das *et al.,* 2013; 2014 revealed 23 and 3.1 g/L from wild grass using used steam-explosion pretreatment and microwave-assisted alkali and organo-pretreatment respectively.

Table 3: Ethanol Titres and Yield Comparison with Previous Studies

S/N	Biomass	Conditions	Ethanol yield (%) and titre (g/L)	Reference
1	Lignocellulosic sugarcane bagasse	*S. cerevisiae*simultaneous hydrolysis and fermentation	0.336 g/L.h; 12.1 g/L	(Fischer *et al.,* 2017)
2	Sugarcane bagasse	Glucose–xylose cofermentation of green liquor–ethanol-pretreated sugarcane bagasse with mixed *S. cerevisiae* strains	92.80%; 68.24 g/L	You *et al*. (2017)
3	Wild grass	Microwave-assisted alkali and organo -pretreatment of wild grass; simultaneous enzyme saccharification and fermentation; Taguchi orthogonal array design	3.1 g/L	(Das *et al.*,2014)
4	Wild grass	Steam explosion of wild grass; simultaneous enzyme saccharification and fermentation	91.6%; 23 g/L	(Das *et al.,* 2013)
5	Sugarcane bagasse	Acid hydrolysis and *S. cerevisiae* fermentation	0.401 g/L.h; 21.47 g/L	**Present Study**

4.0 CONCLUSION

. In conclusions the linear regression model equations developed were able to predict to a high level of confidence, the concentration of the glucose concentration produced during the hydrolysis. A two-level factorial can be used to determine the conditions leading to the efficient pre-treatment of corn stover using acid hydrolysis and result obtained also shows that the fermentation time strongly determines the amount of bioethanol produced.

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