World Journal of Engineering Research and Technology
WJERT
www.wjert.org
SJIF Impact Factor: 5.924

OPTIMIZATION OF GUINEA GRASS PRETREATMENT FOR THE PRODUCTION OF BIOETHANOL USING RESPONSE SURFACE METHODOLOGY

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Article Received on 14/03/2023 **Article Revised on 04/04/2023** **Article Accepted on 24/04/2023**

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ABSTRACT

Increasing demand for energy and shortage of energy have been major challenges facing the world. The development of new technologies for producing fuel from renewable sources is a possible solution to the challenges. In this study, we investigated the optimal conditions for pretreating guinea grass (*Panicum maximum*) used as a feedstock for bioethanol production. Compositional analysis of the sample was performed using the gravimetric method with the following results obtained:
25.8% cellulose, 50.2% hemicellulose, and 24% lignin. The samples were pretreated with a mixed solution of NaOH and $H2SO_4$ at pH = 2–12. The pretreatment was performed at different times, temperatures, and pH values (as designed by design expert 13 software) to assess the optimum point of delignification, meaning the operational conditions that could effectively maximize the enzymatic hydrolysis stage. The reducing sugar yield for each run at different conditions was analyzed using DNS. The optimal yield of the sugar was achieved at a temperature of 120.08℃ and pH of 2.02 after a pretreatment time of 80.56 min. After the validation of the model with the actual experiment at the same conditions, 1109.25 mg/L of reducing sugar was obtained. The high R^2 values of 0.9986 obtained from the model indicated that the fitted models could predict reasonably precise outcomes. The predicted R^2 value of 0.9880 was in reasonable agreement with the adjusted R^2 of 0.9967. Therefore, our findings show that guinea grass meets the requirement as a second-generation bioethanol production feedstock.

KEYWORDS: Guinea grass, Bioethanol, Reducing sugar, DSN, Pretreatment.

1. INTRODUCTION

The demand for energy is currently increasing with the increasing world population, meaning energy demand is higher than its supply, resulting in a global energy crisis. Conventional fuel (fossil), a non-renewable source of energy, can be exhausted without replacement, and its excessive use causes environmental hazards (Klymchuk et al., 2021). As a result, biofuel has attracted considerable scholarly attention owing to its favorable properties, such as renewability, environmental friendliness, and availability.

Bioethanol, a biofuel, is a fermentation product of sugars and carbohydrates produced through biological and chemical conversion of biomass resources, such as crops, municipal solid wastes, and other lignocellulosic materials (Nigam and Singh, 2011). With the reoccurring environmental hazards and envisaged depletion of the fossil reserve, bioethanol has become a promising alternative to petroleum oil (Segovia-Hernández et al., 2022). Bioethanol is exceptionally a premium transportation fuel with superior performance compared with gasoline in several perspectives. For example, ethanol has a higher-octane rating, and its pure form burns more neatly when compared with petroleum oil (Abikoye, 2020; Lynd et al., 2002). The challenge associated with fossil fuels has led researchers to seek alternative sources of clean energy to reduce or eliminate the environmental problems posed by fossil fuels.

Bioethanol is a renewable and carbon-neutral source of energy, and it does not disrupt the equilibrium of air composition of the atmosphere. Bioethanol can be used as a fuel extender, an oxygenate, and an octane enhancer: it is also an important precursor chemical to many industries, such as pharmaceuticals and cosmetics (Sheehan and Himmel, 2001). Bioethanol is currently produced mainly from the fermentation of simple sugars from food crops, such as maize, sugar cane, and sorghum raw materials. At present, Brazil and US are the two leading countries in the production of bioethanol (Medina & Magalhaes, 2021). Brazil and US produce bioethanol from sugar cane and corn, respectively. The bioethanol derived from this substratum class is named first-generation bioethanol (Bertrand et al., 2016).

In 2019, the US produced the largest quantity of bioethanol (15.8 billion gallons), followed by Brazil (8.6 billion gallons), ranking the second largest (Sönnichsen, 2021).

Over the years, feedstocks for bioethanol production have been mainly agricultural-based raw materials, such as sugar crops (sugar cane and fruits) and starch-containing plants (corn,

grains, and potato) (Sandeep and Pinaki, 2016). Currently, attention has been shifted from food-based raw materials (first-generation bioethanol) to lignocellulosic biomass raw materials (second-generation bioethanol). Raposo et al. (2009) reported that sugar beets and molasses could also be feasible for ethanol production. Molasses, an industrial waste, contain a significant amount of heavy metals. As a result, molasses must be pretreated before they can be used for bioethanol production (USDA, 2008). Due to the limitation of molasses, researchers have focused on agricultural starch-based residues and lignocellulosic biomass for the production of bioethanol.

Lignocellulose substances, such as wood chips, bamboo, grasses, Ashoka, and other leaf biomass wastes, are currently being investigated as feedstocks for bioethanol production (Sandeep and Pinaki, 2016).

This present study aimed to contribute to this ongoing initiative in bioethanol production using guinea grass.

Several researchers have used different biomass materials to produce bioethanol. For example, Premjet et al. (2016) used *Achyranthes aspera* and *Sida acuta grasses* to produce bioethanol owing to their high cellulose content. The weeds were pretreated with phosphoric acid (70, 75, and 80%) to improve the enzymatic hydrolysis. The effect of the pretreatment on enzymatic hydrolysis was investigated. The result showed that the conversion of cellulose to glucose was higher in pretreated *A. aspera* (86.2, 0.3%) than in *pretreated S. acute* (82.2, 1.1%).

Alison et al. (2014) investigated the bioethanol potential of Thai grasses. Among the glasses investigated, Tifton Bermuda grass was more promising for bioethanol production owing to its physical properties, such as a high dry matter yield and a high percentage of cellulose.

Arupjyoti et al. (2016) used five invasive weeds: *Saccharum spontaneum, Mikania mikrantha, Lantana camara, Arundo donax, and Eichhornia crasspies,* to produce bioethanol. The yield of total fermentable sugars after the pretreatment and enzymatic hydrolysis of the different grasses was evaluated. The result showed that the average total fermentable sugar yields of all weeds were 43.85 g/100 g raw biomass.

In this study, we chose guinea grass owing to its availability and abundance in Nigeria. Guinea grass is not edible. The transformation of the grass into a high-value product will discourage the burning of the grass, thereby reducing global warming.

We pretreated the guinea grass with a solution prepared with NaOH and H_2SO_4. NaOH and H_2SO_4 were used to adjust the pH of the solution to the desired pH. The optimal pretreatment temperature, pH, and time were investigated, and their effects on the pretreatment were also investigated. Compositional analysis was performed using a gravimetric method to determine the cellulose, hemicellulose, and lignin contents of the sample.

2. MATERIALS AND METHOD

Materials and Reagents

The guinea grass harvested from the University of Benin, Benin City, Ugbowu Campus, Edo State, Nigeria, was used as feedstock materials for this research. NaOH (99.9% pure) produced by CDH, New Delhi, India; cellulase produced by Huazhao Nantong Biotechnology Co Ltd, Nanton, China; H_2SO_4 (98%) produced by LOBA CHEMIE PVT. Ltd, India, were purchased from a local vendor. The reagents were analytical-grade chemicals and were used without further purification. *Saccharomyces cerevisiae* was obtained from the University of Benin Microbiology Laboratory. Distilled water was obtained from Luco Sc. Laboratory, Benin City.

Experimental design

Experimental runs were designed using Design Expert® (version 13.0 demo, Stat-Ease, Minneapolis, MN, USA). Box–Benkhen design (BBD) was used for all experiments. Fermentable sugars of the treated grasses were analyzed, which were the response in all pretreatments. Mathematical models generated with BBD were used to predict fermentable sugars for each sample. Below is the experiment design for this work.

Table 1: Variables with their range of values for the pretreatment yield.

Independent variable	Symbol	Levels	
		-1	+1
Temperature (℃)	X_1	60	121
Time (mins)	X_2	60	120
pH	X_3	2	12

Table 2: Experimental numbers of runs.

	Factor 1	Factor 2	Factor 3
Run	A: pH	B: Time (min)	Temperature ℃
1	2	60	90.5
2	7	90	90.5
3	12	90	121
4	12	60	90.5
5	7	90	90.5
6	7	60	60
7	7	90	90.5
8	7	90	90.5
9	2	90	121
10	2	90	60
11	12	90	60
12	7	90	90.5
13	7	120	121
14	12	120	90.5
15	2	120	90.5
16	7	60	121
17	7	120	60

Compositional analysis

The composition analysis was performed using the gravimetric method (Carrier et al., 2011) to determine the lignin, cellulose, and hemicellulose contents of the guinea grass.

About 10 g of ground biomass was added to 500 mL of 0.5 M NaOH solution, and the mixture was boiled at 100℃ for 2 h to remove the hemicellulose content. Afterward, the sample was allowed to stand and washed with distilled water until the pH of the sample became 7. The sample was dried in an oven at 105^{o}C. The dried sample was weighed to determine hemicellulose content. Then, 500 mL of 4 M H_2SO_4 was added to the dried sample and boiled for 2 h, and it was allowed to stand for 24 h at ambient temperature. Afterward, the mixture was decanted, and the undissolved residue was washed with water several times until pH 7 was achieved. The residue was dried at 105^{o}C in the oven for 4 h, cooled in a desiccator, and weighed to determine lignin.

The cellulose content of the sample was measured using Equation 1.

$$C_m = B_m - H_m - L_m \quad (1)$$

where;

C_m = *mass of cellulose content*

B_m = *mass of biomass 10 g*

L_m = *mass of lignin*

H_m = *mass of hemicellulose*

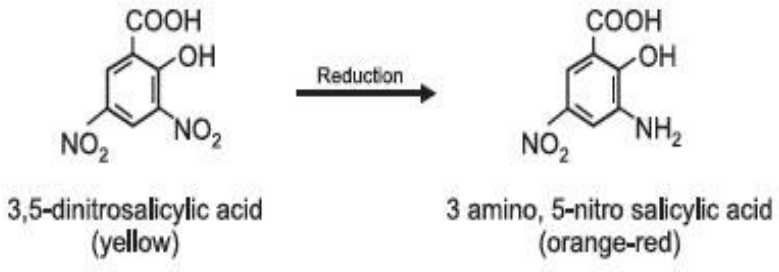
Pretreatment of the feedstocks

To expose the cell wall of the guinea grasses and influence their cellulose, hemicellulose, and lignin content, they were sliced and dried in the oven at 70℃ temperature for 3 d and then crushed in a mill and sieved. The crushed sample sizes were less than 100 µm. Precisely, 10 g of the samples were weighed using an electrical weighing balance (ATOM A 110C). Afterward, the sample was transferred to a conical flask containing 150 mL of the mixed solution (NaOH and H_2SO_4) with different pH values. NaOH and H_2SO_4 were used to regulate the pH of the mixed solution to achieve the desired pH values ranging from 2 to 12. Then, the conical flasks containing the samples of different pH values were autoclaved at different temperatures ranging from 60 to 121^oC following the experiment design procedure. After the samples were autoclaved, they were neutralized to achieve pH 7. Afterward, distilled water was added to the conical flasks to make up the volume of the samples to 250 ml.

Estimation of reducing sugars using the dinitro salicylic acid (DNS) method

Preparation of DNS solution

Reducing sugars have the ability to reduce many of the reagents (Deshmukh and Madhukar, 2020). One of the reagents, such as 3,5-DNS in an alkaline solution, can be reduced to 3 amino 5 nitro salicylic acid.



Scheme1: Reduction of 3, 5-DNS (Deshmukh and Madhukar, 2020).

Sodium potassium tartrate (45 mg) was dissolved in 75 mL of H_2O, and 80 mg of 2 M NaOH was dissolved in 1 liter of water. Then, 1.5 gm of 3,5-DNS reagent was dissolved in 30 mL of NaOH solution. The resultant solution was added to sodium potassium tartrate solution. Afterward, distilled water was added to the mixed solution to make its volume up to 150 mL.

Preparation of standard sugar solution

The stock standard sugar solution was prepared by dissolving 250 mg of glucose in 100 mL of distilled water. Then, 75, 50, 30, 20, and 10 mL of distilled water were added to 25, 50, 30, 20, and 10 mL of the prepared stock solution to obtain a standard glucose solution (1000, 750, 500, 300, 200, and 100 mg/L).

Estimation of sugar contents

The sugar content of the samples was determined following the reported procedure of Deshmukh and Madhukar (2020). After the samples were autoclaved and diluted with distilled water, a small amount of the samples were collected and filtered with filter paper. Then, 3 mL of the filtrates were separately collected and transferred to test tubes, and 1 mL of DNS solutions was added and boiled for 5 min at 100℃. Then, 5 mL of distilled water was added to the mixtures and allowed to cool at room temperature. The absorbances of the mixture were read via a UV meter at the wavelength of 540 cm^{-1}. The corresponding values of sugar concentration were calibrated using a standard glucose solution of 1000, 750, 500, 300, 200, and 100 mg/L.

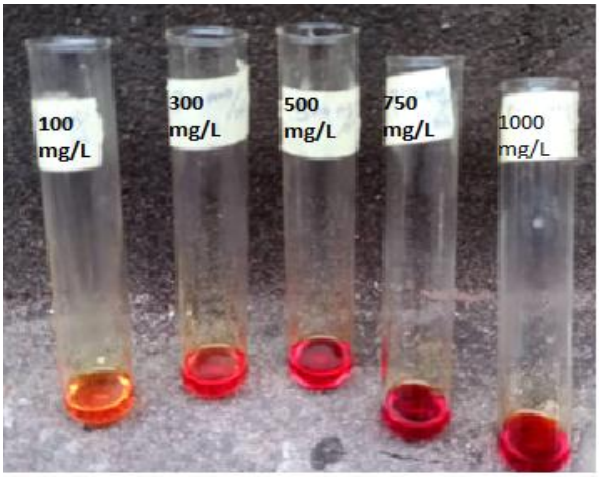


Figure 1: Analysis of reducing sugar in standard sugar solution using DNS.

Enzymatic hydrolysis

Prior to the hydrolysis of the pretreated sample, NaOH was added to raise the pH from 2 to 7 in order to create a safe environment for the enzymes. Then, cellulase was added and incubated for 24 h at 45^{o}C temperature. After 24 h of incubation, the sugar content of the hydrolysate was measured using ultraviolet-visible spectroscopy.

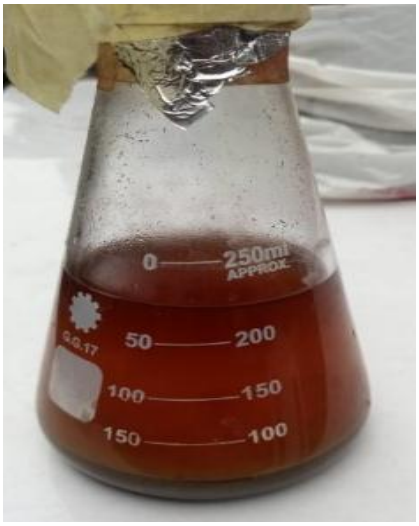


Figure 2: Enzymatic hydrolysis of pretreated grass.

Ethanol fermentation

Saccharomyces cerevisiae was used for the fermentation of the hydrolyzed sugar. The first reading was taken after 8 h, and the subsequent readings were taken for 24 h using a refractometer.

Characterization of the biomass

The gravimetric method was used to analyze the cellulose, hemicellulose, and lignin contents of the biomass (guinea grass). The following results were obtained 25.8%, 50.2%, and 24% of cellulose, hemicellulose, and lignin, respectively. The results were consistent with the previously reported results.

Table 1: concentration of reducing sugar present in each pretreated sample.

Run	A: pH	B: Time (min)	Temperature (℃)	Reducing concentration mg/L
1	2	60	90.5	1053.82
2	7	90	90.5	529.80
3	12	90	121	459.26
4	12	60	90.5	450.19
5	7	90	90.5	560.03
6	7	60	60	408.87
7	7	90	90.5	549.95
8	7	90	90.5	494.53
9	2	90	121	1361.18
10	2	90	60	1053.82
11	12	90	60	524.76
12	7	90	90.5	519.72
13	7	120	121	388.72
14	12	120	90.5	423.99

15	2	120	90.5	1079.01
16	7	60	121	479.41
17	7	120	60	548.44

Table 1 shows the reducing sugar concentrations of the pretreated grass under different conditions. From the table, run 9 yielded a high concentration of sugar. The optimal concentration of the reducing sugar predicted by the DOE software was 1366 mg/L at the optimal pH, temperature, and time conditions, which were 2.02, 80.56 min, and 120.08 ℃ The optimal-predicted conditions were validated with the actual experiment, and the actual concentration yield of the reducing sugar was found to be 1109.25 mg/L.

Enzymatic hydrolysis

The sample of the pretreated grass was hydrolyzed using cellulase at optimal conditions. The reducing sugar concentration after the hydrolysis was 1109.25 mg/L

Analysis of pretreatment yield

The concentration yields of the pretreated guinea grass in terms of actual and predicted values are also given in Table 1.

Table 2: Experimental design matrix of the pretreated guinea grass at different factors.

Run order	Coded factors			Actual factors			Pretreatment yield	
	A	B	C	pH	Time (min)	Temperature (℃)	Actual value	Predicted value
1	-1	-1	0	2	60	90.5	1081.71	1092.63
2	0	0	0	7	90	90.5	529.80	538.06
3	1.	0	1	12	90	121	443.10	441.06
4	1	-1	0	12	60	90.5	450.19	460.33
5	0	0	0	7	90	90.5	560.03	538.06
6	0	-1	-1	7	60	60	408.87	395.92
7	0	0	0	7	90	90.5	549.95	538.06
8	0	0	0	7	90	90.5	530.80	538.06
9	-1	0	1	2	90	121	1361.18	1358.37
10	-1	0	-1	2	90	60	1053.82	1055.85
11	1	0	-1	12	90	60	670.26	673.07
12	0	0	0	7	90	90.5	519.72	538.06
13	0	1	1	7	120	121	388.72	401.67
14	1	1	0	12	120	90.5	423.99	413.07
15	-1	1	0	2	120	90.5	1091.01	1080.87
16	0	-1	1	7	60	121	600.00	591.89
17	0	1	-1	7	120	60	519.01	527.12

Model summary statistics

The model summary statistics of reducing sugar yield are given in Table 2

Table 3: Model summary of statistics of reducing sugar yield.

Source	Std. Dev	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	204.44	0.6099	0.5198	0.2337	1.067E+06	
2FI	211.14	0.6799	0.4879	-0.4352	1.999E+06	
Quadratic	**16.97**	**0.9986**	**0.9967**	**0.9880**	**16651.02**	**Suggested**
Cubic	16.44	0.9992	0.9969		*	Aliased

The coefficient of determination (R^2) value is a statistical measure representing the proportion of the variance for a dependent variable explained by an independent variable or variables. The R2 value measures how variability in the observed response values can be explained by the experimental factors and their interactions. (Ying *et al*., 2011). R^2 value closer to unity gives a better representation of a process. From Table 3, the cubic source of the model gives a better R^2 value. However, cubic models are aliased from response surface methods with the assumption that the effects of cubic factors are highly insignificant, making the quadratic with a better R^2 value than the two-factor interaction (2FI) and linear source the best representation of the process. A strong and close correlation was observed between the adjusted and predicted R^2 values of 0.9986 and 0.9967 (difference less than 20%) of the reducing sugar, which indicates that the process's experimental and predicted values are in reasonable agreement. Figure 3 shows the close correlation between the predicted value and the actual value.

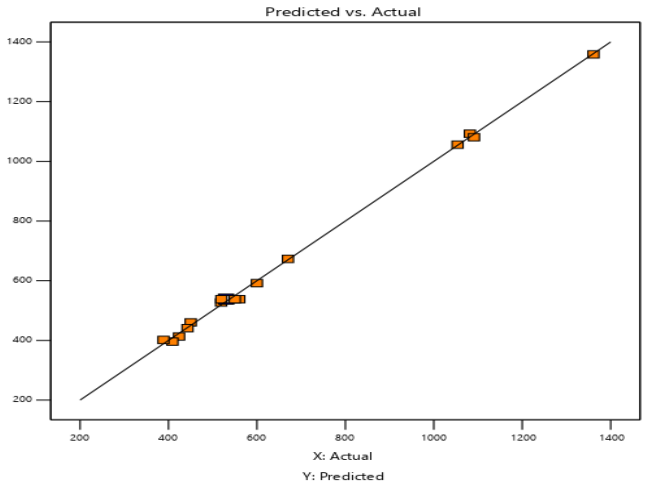


Figure 3: Predicted vs. actual plot of reducing sugar yield.

Table 4: Analysis of variance (Anova).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	$1.391x10^6$	9	$1.545x10^5$	536.42	< 0.0001	significant
A-pH	$8.451x10^5$	1	$8.451x10^5$	2933.81	< 0.0001	
B-Time	1741.73	1	1741.73	6.05	0.0435	
C-Temperature	2485.94	1	2485.94	8.63	0.0218	
AB	315.08	1	315.08	1.09	0.3304	
AC	71429.44	1	71429.44	247.96	< 0.0001	
BC	25828.96	1	25828.96	89.66	< 0.0001	
A^2	$4.133 x10^5$	1	$4.133 x10^5$	1434.74	< 0.0001	
B^2	33832.01	1	33832.01	117.45	< 0.0001	
C^2	3975.40	1	3975.40	13.80	0.0075	
Residual	2016.44	7	288.06			
Lack of Fit	935.09	3	311.70	1.15	0.4302	not significant
Pure Error	1081.35	4	270.34			
Cor Total	$1.393 x10^6$	16				

Table 4 shows the ANOVA for the pretreatment of guinea grass and the significance of the process variables on the reducing sugar yield. The ANOVA was performed based on a significant level of 5%, i.e., the probability value of 0.05 ($\alpha = 0.05$).

The **model F-value** of 536.42 implies that the model is significant. There is only a 0.01% chance that a large F-value can occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case, A, B, C, AC, BC, A^2, B^2, and C^2 are significant model terms. Values greater than 0.1000 indicate that the model terms are insignificant.

The F-value of 1.15 for lack of fit indicates that the lack of fit is insignificant relative to the pure error. There is a 43.02 % possibility that a significant lack of fit F-value is caused by noise. Thus, an insignificant lack of fit is acceptable.

Regression model

The empirical relationships between the guinea grass pretreatment variables considered and the reducing sugar yield are given by equation (4.1) in coded forms.

Reducing sugar yield = 538.062 + -325.023 * A + -14.7552 * B + 17.6279 * C + -8.87527 * AB + -133.631 * AC + -80.357 * BC + 313.301 * A^2+ -89.6387 * B^2+ 30.7272 * C^2(4.1)

In a regression equation, when an independent variable has a positive sign, an increase in the variable will cause an increase in the response, while a negative sign will decrease the

response (Russel, 2009; Ocholi *et al*., 2018). From the model in equation (4.1), temperature (C) and quadratic factors (A^2 and C^2) have positive effects on the yield of reducing sugar, while pH (A), time (B), interaction factors (AB, AC, and BC), and quadratic factor (B^2) have negative effects on the concentration yield of reducing sugar.

Table 5: Fit Statistics pretreatment process models.**

Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	
Linear	0.0054	< 0.0001	0.5198	0.2337	
2FI	0.5575	< 0.0001	0.4879	-0.4352	
Quadratic	**< 0.0001**	**0.4302**	**0.9967**	**0.9880**	**Suggested**
Cubic	0.4302		0.9969		**Aliased**

Table 6: Fit Statistics of the pretreatment process model.

Std. Dev.	16.97	**R^2**	0.9986
Mean	657.77	Adjusted R^2	0.9967
coefficient of variation (CV) %	2.58	Predicted R^2	0.9880
		Adeq Precision	73.9367

From Table 4.6, a CV value of 2.58 for the pretreatment of guinea grass to reducing sugar is within an acceptable range. Because the CV represents standard deviation as a percentage of the mean, a smaller CV value gives better reproducibility. Generally, a high CV indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model (Daniel, 1991; Liyana-Pathirana and Shahidi, 2005). The suitability of the models was tested using the coefficient of determination (R^2). The high values of R^2 (0.9986) for the pretreatments indicate that the fitted models can be used to predict reasonably precise outcomes (Suwanthai *et al*., 2016; Chumuang and Punsuvon, 2017). The predicted R^2 value (0.9880) is in reasonable agreement with the adjusted R^2 of 0.9967; i.e., the difference is less than 0.2. Adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 73.937 indicates an adequate signal for the guinea pretreatment. This model can be used to navigate the design space.

Effects of interaction variables of reducing sugar yield

The effects of the process variables on the concentration yield of reducing sugar were studied by plotting three-dimensional surface curves against any two independent variables while keeping the other variables constant at their central (0) level (Millika, Chanpim, and Alissara, 2020; Chumuang and Punsuvon, 2017) **(**Figures 4, 5, and 6).

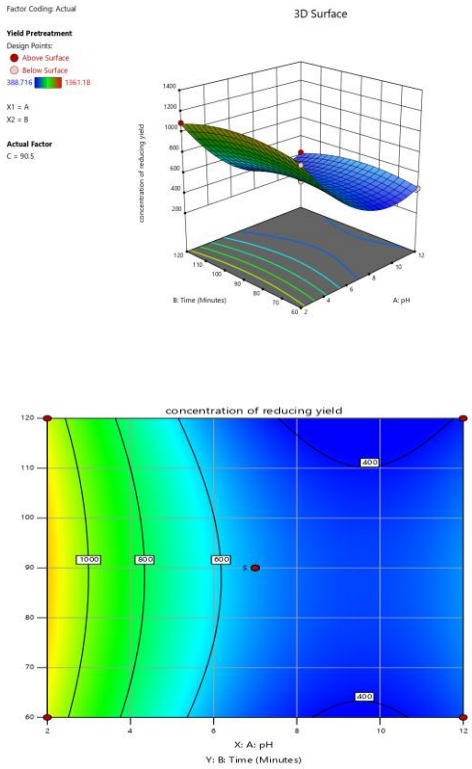


Figure 4: Interaction effects of variables on concentration yield of reducing: pH and time.

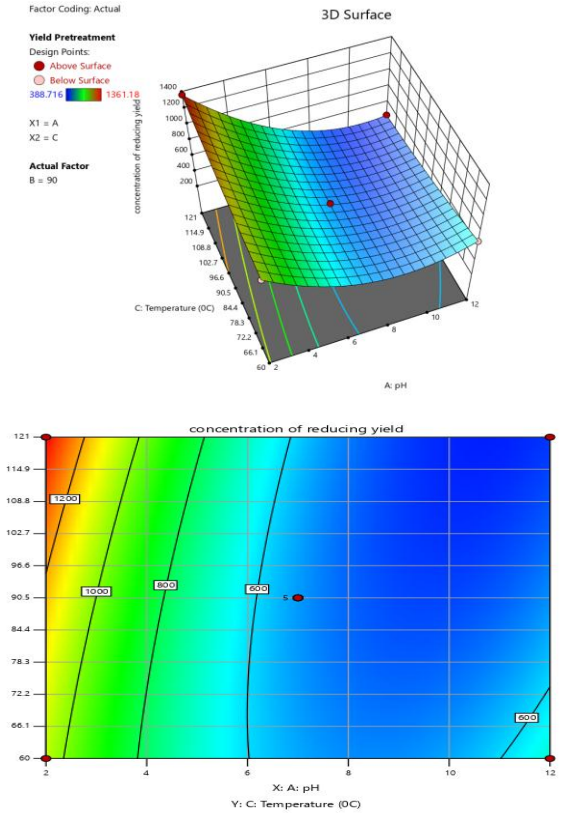


Figure 5: Interaction effects of variables on the yield of reducing sugar: pH and temperature.

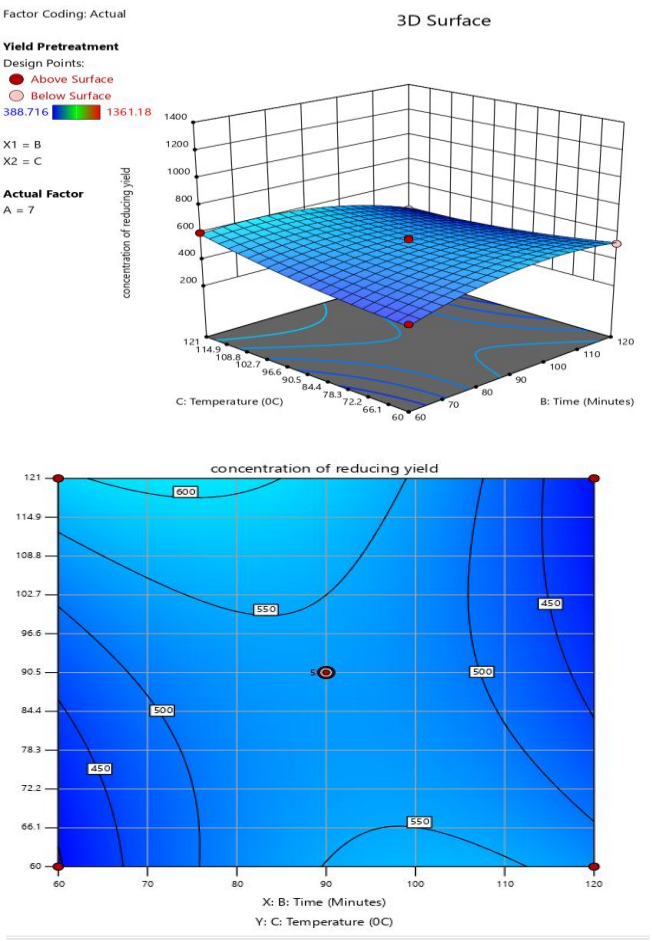


Figure 6: Interaction effects of variables on concentration yield of reducing: time and temperature.

The interaction effects of time and pH (AB) on the yield of reducing sugar are shown in Figure 4. At constant temperature, the increase in time and pH leads to a slight increase in the concentration yield of reducing sugar. Figure 5 shows the interaction effects of temperature and pH concentration yield of reducing sugar at constant time. An increase in temperature and decrease in pH increased the concentration yield of reducing sugar. Figure 6 shows the interaction effects of temperature and time on the concentration yield of reducing sugar. The simultaneous increase in temperature and time increased the concentration of reducing sugar.

Optimization of reducing sugar yield

From the numerical optimization studies carried out on the pretreatment process, the maximum desirability of 1 with three factors (pH, temperature, and time) shows that it is possible to achieve a maximum reducing sugar yield of 100% desirability. The optimal predicted concentration yield of reducing sugar (1366 mg/L) was obtained at a temperature of 120.08℃ and pH of 2.02 after a pretreatment time of 80.56 mins; after the validation of the

model with actual experiment at the same conditions, a concentration yield of 1109.25 mg/L was obtained.

Ethanol production potentials of guinea grass

S. cerevisiae yeast strain was used to ferment the hydrolysate obtained from the enzymatic hydrolysis to evaluate the ethanol production potential of the guinea grass via the Separate hydrolysis and fermentation technique.

After the fermentation of the sample containing 1109.25 mg/L, the following results of bioethanol were obtained: 9.8, 8.3, 9.5, and 10 % after 8, 24, 48, and 72 h, respectively. The maximum ethanol yield was 10% after 48 h of fermentation. The variation in the results can be attributed to a drop in the fermentation temperature.

Characterization of the biomass

The following results were obtained 25.8, 50.2, and 24% for cellulose, hemicellulose, and lignin, respectively, after the composition analysis of guinea grass. The results were consistent with those previously reported (Premjet et al., 2016).

3. CONCLUSIONS AND RECOMMENDATIONS

Conclusion

The following conclusions were drawn from this study:

1. Guinea grass delivered satisfactory results as a second-generation feedstock for ethanol production.
2. The optimal concentration yield (1109.25) of reducing sugar was obtained at a temperature of 120.08℃ and pH of 2.02 after a pretreatment time of 80.56 min
3. The maximum conversion of the sugar to bioethanol was obtained after 24 h of fermentation.
4. The raw guinea grass comprised 25.8% cellulose, 50.2% hemicellulose, and 24% lignin.
5. The pH had the most significant influence on the pretreatment of guinea grass.
6. The high values of R^2 of 0.9986 indicated that the fitted models could predict reasonably precise outcomes. The predicted R^2 of 0.9880 was in reasonable agreement with the adjusted R^2 of 0.9967.

Recommendation

The following are recommended for further studies:

1. The enzymatic hydrolysis and fermentation of the sample should be optimized to know the best-operating conditions of the enzymes and the yeast.
2. The inhibition of the process should be considered in order to achieve the best results.

Undertaking

No part of this manuscript has been published elsewhere (except in the form of an academic thesis).

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