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Optimizing the Conversion of Pretreated Sila Sorghum Stalks to Simple Sugars Using Immobilized Enzymes

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Abstract - *Sila sorghum stalks are agricultural wastes found in Kenya which can be put into profitable use by replacing food based substrates in the production of simple sugars leading to food security while at the same time help address land and air pollution in cases where they are dumped on land and burnt during land preparation. Sila sorghum stalks were investigated for their potential to produce simple sugars. The stalks were dried and milled into fine powder followed by alkali pretreatment then hydrolysis using immobilized enzymes. The parameters studied were temperature, pH and concentration of sodium alginate (NaAl). The level of temperature was varied at 40 - 70°C, pH at 4.5 - 7.5, while concentration of NaAl was varied at 1.0% - 3.0% (w/v). Hydrolysis time was 48 hours. The maximum hydrolysis yield was established using a central composite rotatable design (CCRD) in response to surface methodology (RSM). Mathematical models estimating the yield of simple sugars from pretreated Sila sorghum stalks were developed and analyzed for predicting the yield of simple sugars. Optimum glucose yield of 71.3% (w/w) was obtained at 55°C hydrolysis temperature, pH of 6.0 and 2.0 % concentration of sodium alginate (w/v). Lowest glucose yield of 35.5 % was obtained at 55°C, pH of 6.0 and 3.7% concentration of sodium alginate. The yield of glucose obtained from the hydrolysis of pretreated Sila sorghum stalks (71.3%, w/w) using immobilized enzymes indicate that Sila sorghum stalks are suitable for the production of simple sugars which can be used to produce bioethanol and marketable chemicals such as citric and lactic acid.*

Key words: Bioethanol, Central Composite Rotatable Design (CCRD), Glucose, immobilized, Sila sorghum stalks,

1. INTRODUCTION

The demand for simple sugars globally is on the rise since they are used as raw material for many industrial chemicals such as citric and lactic acid. Currently the available substrates (cereals, sugar and starch) for the production of simple sugars are mainly used as food sources. Secondly, the need to find alternative, renewable and sustainable sources of energy has led to increased attention towards biofuels such as bioethanol which is produced from fermentation of simple sugars. On the other hand, there are several initiatives to enhance dairy farming in Kenya by providing nutritious animal feed to dairy animals. Candidate animal feeds can be enriched using simple sugars to increase their energy content. In order to address these requirements, more research is required to find out viable, renewable and sustainable sources of simple sugars suitable for the production of bioethanol which is a renewable source of energy and industrial chemicals such as citric acid and lactic acid as well as simple sugar concentrates for enriching animal feeds thereby enhancing dairy production. Currently, research on non-food crops such as lignocellulosic biomass (LGB) as a source of simple sugars is attracting great attention worldwide because they are cheap, abundant, sustainable and profitable when compared to substrates such as cereals, sugar and starch. Substrates being considered include agro-wastes (corn stover, wheat straws, sugar cane bagasse, and sorghum stalks), hardwood and softwood residues from forest resources [6]. The conversion of LGB to bioethanol and other valuable chemicals involves the liberation of simple sugars from biomass substrates which consist of three fractions (hemicellulose, cellulose and lignin). Hemicellulose and cellulose contain sugars in polymeric form that can be converted by enzymes, alkali or acids to simple sugars. However, the lignin component shields cellulose and hemicellulose from microbial and enzymatic attack. According to [10], pretreatment of LGB is required in order to make LGB easily accessible to enzymatic saccharification. Even though several attempts have been made to produce simple sugars from non-food substrates such as LGB, the conversion of these substrates to simple sugars is often faced with several draw backs. The lack of sustainable non-food substrates presents a challenge to the commercialization of processes for the production of simple sugars from LGB. In an attempt to address these challenges, this research study sought to develop a process of enzymatic hydrolysis of pretreated Sila sorghum stalks to simple sugars using immobilized enzymes. Despite the wide interests in the production of simple sugars from agricultural residues, the hydrolysis of Sila sorghum stalks using immobilized enzymes has not been reported in literature. Furthermore, this LGB has never been explored as an alternative substrate for the production of simple sugars using immobilized enzymes in Kenya. According to [11], it is possible to hydrolyze woody biomass to simple sugars using immobilized enzymes. In order to widen the scope of using this method of hydrolysis, it is necessary to test the same on agrowastes such as Sila sorghum stalks. This will provide information on the applicability of this method of hydrolysis on a wide variety of lignocellulosic substrates.

2. MATERIALS AND METHODS

2.1 Enzymes and reagents

The enzymes used in this study were cellulase and cellobiase (β glucosidase). The reagents included calcium chloride, sodium chloride, glucose standard, glutaraldehyde (GA), acetone, glucose hexokinase (HK), sodium hydroxide, deionised water, sodium azide, sodium alginate (NaAl) and sulphuric acid [11].

2.2 Design of experiment

Three variables (temperature, pH and concentration of NaAl used in immobilizing cellobiase) were studied, each at five levels inclusive of star points. MATLAB was used in the design of the experiment leading to the generation of the experimental design matrix (Table 3) which shows the actual and coded level of factors [11].

Table - 1: Coded factor levels and actual values of variables

		Levels				
Variables	Notation	- α	-1	0	+1	+ α
Temp, (°C)	X1	29.8	40	55	70	80.2
pH	X2	3.5	4.5	6	7.5	8.5
Conc. NaAl	X3	0.32	1	2	3	3.7

2.3 Collection of samples

Sila sorghum stalks samples were collected from a farm situated in Bungoma County after harvesting the sorghum grain. The samples were stored in plastic bags and then dispatched to the Chemical and Process Engineering laboratory.

2.4 Drying and milling

The Sila sorghum stalks (substrate) were washed several times with tap water to remove any adhering dirt and sun dried for two days. The stalks were then cut into 2cm sized pieces and dried using a hot air oven set at 105°C for 8 hours. The oven dried Sila sorghum stalks were then milled through a 0.3 mm screen into fine powder and stored in sealed plastic containers [11]. The chemical composition of Sila sorghum stalks was analyzed using procedures described by [2], [7], [8] and [9].

2.5 Pretreatment of substrate

This method was based on [11, 12]. 40 g of substrate milled to an average particle size of 0.3 mm was soaked in sodium hydroxide (0.1 M NaOH) solution at 10% solid loading for 24 hours at room temperature. The slurry was then filtered through a sieve to separate solid and liquid fractions. The solids were thoroughly washed with distilled water to remove the residual NaOH. The washed solid was then dried at 60°C for 6 hours and stored in a sealed container for use in the hydrolysis experiment [11].

2.6 Enzyme immobilization

This procedure was similar to the one used by [11]. 10% (w/w) enzyme loading of cellobiase was mixed with sodium alginate (NaAl) solution to form separate immobilizing mixture. The concentration of NaAl was set depending on the experiment as determined by the CCRD. The beads were formed by dripping the enzyme-polymer solution into stirred 0.2 M calcium chloride ($CaCl_2$) solution using a syringe. The beads were left in $CaCl_2$ solution to cure for 30 minutes and then washed using distilled water and finally stored at 4°C for use in further experiments [4]. To immobilize cellulase an enzyme loading of 10% (w/w) was dissolved in 10 ml of 0.1 M citric acid buffer. Acetone was added into a 50 ml glass vial with a magnetic stirrer. The enzyme-buffer mixture was then added to the acetone. GA was then added drop wise to give a solution with a concentration of 0.005 M which was maintained at 10°C for 2 hours with gentle stirring [5]. The mixture was finally centrifuged using a centrifuge machine for 15 minutes and the immobilized cellulase enzyme pellets obtained upon decanting and washing them using buffer solution. The immobilized cellulase enzyme system was stored in buffer solution at 4°C for use in subsequent hydrolysis experiments [5].

2.7 Optimal levels of factors affecting hydrolysis

The method used was based on [3]. 60 separate pretreated substrate samples equivalent of 0.1 g of cellulose were weighed out and added to 20 ml scintillation vials. 5.0 ml sodium acetate buffer at the pH level determined by the experimental run was then added to each sample to form separate experiment samples. Each vial was placed in a rotary shaker which was operated at 150 rpm for 30 min after which the vial was placed in a water bath set at the temperature level determined by the experimental run. To initiate the reaction, 4.0 ml immobilized enzyme was added to the substrate samples after reaching the desired reaction temperature as per the experimental design. Hydrolysis time was 48 hours and samples to be analyzed for glucose were stored at -4°C prior to analysis. Glucose analysis was based on [1]. Each experimental treatment was performed in triplicate and the average response calculated for each experimental run.

2.8 Statistical analysis

Coefficient of determination (R^2) and adjusted R^2 were used to determine the significance of the developed models. The double tailed student's T-test at 95% confidence level was applied to test the significance of the various polynomial

coefficients. Fischer test at 95% confidence level was applied to test the statistical significance of the developed polynomial equations. The statistical analyses and plotting of contour and surface plots were done on MATLAB version R2010b [11].

3. RESULTS AND DISCUSSION

3.1 Materials Characterization

Characterization was done to determine the chemical composition of Sila sorghum stalks. The summary of the compositions of Sila sorghum stalks is as shown in table-2.

Table - 2: Chemical composition of Sila sorghum stalks

Component	%
Cellulose	27.04
Hemicellulose	27
Lignin	31.28
Ash	4.7
Moisture	9.45
Extractives	0.53
TOTAL	**100**

In LGB, lignin offers protection to the plant against chemical and biological attack and must be removed during pretreatment in order for the enzymes to access the cellulose and break it to glucose [12]. The high amount of lignin (31.28%) found in Sila sorghum stalks is as a result of the need to provide structural support to the Sila sorghum stalks during growth. The removal of lignin needs to be effective and in order to achieve this; the substrate was soaked in sodium hydroxide (0.1 M NaOH) solution at 10% solid loading for 24 hours at room temperature so as to pretreat it. Alkali treatment dissolves most of the lignin and hemicellulose since the two components are highly associated with each other leaving behind a less crystalline, highly cellulose rich substrate which is easily susceptible to enzymatic hydrolysis. Reference [13] reported that enzymatic hydrolysis was greatly improved when lignin removal during pretreatment was increased because a larger surface area of cellulose was exposed to hydrolyzing enzymes leading to high yield.

3.2 Optimal Hydrolysis Conditions

3.2.1 Glucose yield from Sila sorghum stalk

Table -3 shows the experimental design matrix with the actual and predicted percentage yield of glucose obtained in each experimental run. Under the experimental conditions applied in this study, the maximum glucose yield was obtained in experimental run 17 (71.3%, w/w). This was achieved at a temperature of 55°C, pH of 6.0 and a 2.0% (w/v) concentration of sodium alginate. These levels of factors were considered to be the optimum.

Table -3: Glucose yield from pretreated Sila sorghum stalks: Actual and Predicted

RUN	X1	X2	X3	Glucose yield (%, w/w) (Actual)	Glucose yield (%, w/w) (Predicted)
1	40	4.5	1.0	51.3	51.5
2	70	4.5	1.0	60.4	61.7
3	40	7.5	1.0	52.1	52.9
4	70	7.5	1.0	62.8	63.1
5	40	4.5	3.0	42.6	42.3
6	70	4.5	3.0	48.2	49.4
7	40	7.5	3.0	43.1	43.7
8	70	7.5	3.0	51.2	50.9
9	29.8	6.0	2.0	44.2	43.9
10	80.2	6.0	2.0	59.6	58.6
11	55	3.5	2.0	63.3	62.4
12	55	8.5	2.0	65.2	64.8
13	55	6.0	0.32	54.3	53.2
14	55	6.0	3.7	35.5	35.2
15	55	6.0	2.0	71.1	71.0
16	55	6.0	2.0	70.9	71.0
17	55	6.0	2.0	71.3	71.0
18	55	6.0	2.0	70.8	71.0
19	55	6.0	2.0	70.9	71.0
20	55	6.0	2.0	70.7	71.0

Equation 1 below is a 10 terms, 2nd order regression polynomials which was fitted to predict the yield of glucose from the hydrolysis of pretreated Sila sorghum stalks.

$$Y=70.9879+4.3492X1+0.7245X2-5.3537X3+0.5125X1X2-0.7625X1X3+0.0375X2X3-6.9820X^2 1-2.6167\ X^2 2-9.4563\ X^2 3+\epsilon_{Gluc} \quad \ldots\ldots (1)$$

Where

Y=Predicted glucose yield from pretreated Sila sorghum stalks (%, w/w)

ϵ_{Gluc} = Random error associated with glucose yield prediction from pretreated Sila sorghum stalks

Xi = Dimensionless values of the independent variable

The analysis of a double – tailed student T- test at 95% confidence level was done using MATLAB and the results are as shown in (Table 4).

Table -4: Sila sorghum stalks: Test of significance of the various regression coefficients, **t-critical=2.228**

Term	Se (β_i)	t_o	p- Value	Comment
β_0	0.3173	223.7	7.83E-20	Significant
β_1	0.2105	20.658	1.56E-09	Significant
β_2	0.2105	3.4414	0.006316	Significant
β_3	0.2105	-25.429	2.03E-10	Significant
β_{12}	0.2750	1.8631	0.09205	Insignificant
β_{13}	0.2750	-2.7719	0.01972	Significant
β_{23}	0.2750	0.13632	0.89427	Insignificant
β_{11}	0.2049	-34.072	1.12E-11	Significant
β_{22}	0.2049	-12.769	1.63E-07	Significant
β_{33}	0.2049	-46.146	5.50E-13	Significant

From (table -4), regression coefficients associated with interaction terms X_1 and X_2, and X_2 and X_3 were insignificant and were hence collated with other errors as ϵ_{Gluc} and a reduced model for the yield of glucose from pretreated Sila sorghum stalks was obtained as equation 2.

$$Y=70.9879+4.3492X1+0.7245X2-5.3537X3-0.7625X1X3-6.9820X^21-2.6167X^22-9.4563X^23+\epsilon_{Gluc} \quad (2)$$

Where

Y=Predicted glucose yield from pretreated Sila sorghum stalks (%, w/w)

ϵ_{Gluc} = Random error associated with glucose yield prediction from pretreated Sila sorghum stalks

Xi = Dimensionless values of the independent variable

The reduced regression model (equation 2) was used to plot response surface graph as shown in chart-1 and for prediction of response. The values of predicted glucose yield are shown in table- 3.

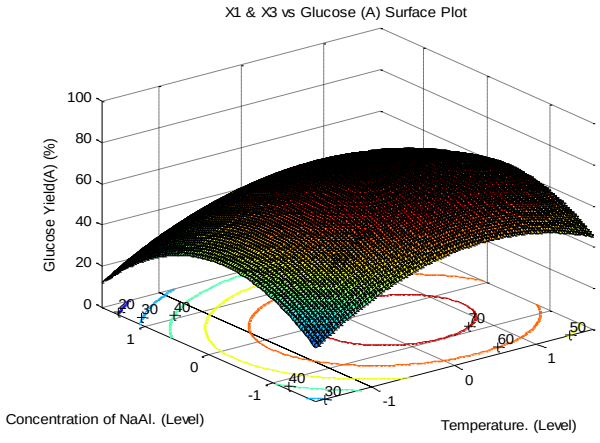

Chart -1: Pretreated Sila sorghum stalks: Glucose (A) vs X_1, X_3: Response surface and contour plot

Chart-1 was plotted using MATLAB R2010b. The plot shows a response surface with contours depicting how both temperature and concentration of sodium alginate influenced the yield of glucose. The plot shows that the maximum yield of glucose (71.3%) from pretreated Sila sorghum stalks is obtained when temperature and concentration of sodium alginate are 55°C (0) and 2.0% (0) respectively, that is both factors at the central setting. This is illustrated by the smallest eclipse on the contour plot. The results indicated by the plot are in agreement with the experimental observations which showed that a temperature of 55°C and a 2.0% concentration of sodium alginate led to optimum yield of glucose from pretreated Sila sorghum stalks [11]. ANOVA was performed using MATLAB R2010b to test the significance of the model depicting the yield of glucose from pretreated Sila sorghum stalks, and the results are shown in (Table- 5).

Table- 5: Pretreated Sila sorghum**:** ANOVA for the regressions significance, **F- test $_{(critical)}$ =3.02, p value - Prob > F =0**

Source	Sum of Squares	df	Mean of Squares	F-test (observed)
Model	2503.20	9	278.133	459.436
Error	6.053	10	0.6054	
Total	2509.26	19		

From (Table- 5) the model representing the yield of glucose from pretreated Sila sorghum stalks has an F- value of 459.4368 which is higher than the critical F – value of 3.02 at 95% confidence level. This shows that the developed model equation 1 significantly evaluates the experimental data involving glucose yields from pretreated Sila sorghum stalks. The model F – value (459.4368) implied that the model was significant and there was negligible (0.00%) chance that an F- value this large can occur because of noise. In addition, from regression analyses, the coefficient of determination, R^2 for the model is 0.9976 with an adjusted R^2 of 0.9954 for glucose which indicates that the developed model is significant. This implies that there is a better correlation between observed and predicted values of responses. The results obtained in this study are in agreement with the ones obtained in [11]. Similar hydrolysis trends were obtained when using pretreated *Prosopis Juliflora* stem as substrate. The difference in glucose yield can be attributed to the difference in composition of substrate and the extent of pretreatment realized. Reference [5] used microcrystalline cellulose (pure form of cellulose) during their study on hydrolysis of cellulose using immobilized enzymes. The current study proposes a suitable process involving enzymatic hydrolysis of pretreated Sila sorghum stalks

(natural form of LGB) using immobilized cellulase and cellobiase.

4 CONCLUSIONS

The aim of this research study was to establish whether pretreated Sila sorghum stalks can produce substantial quantities of simple sugars through hydrolysis using immobilized enzymes and the optimum level of hydrolysis conditions. The following conclusions were drawn from the study:

a. Optimum glucose yield of 71.3% (w/w) from pretreated Sila sorghum stalks were obtained at 55°C hydrolysis temperature, pH of 6.0 and 2.0% concentration of sodium alginate.
b. Pretreated Sila sorghum stalks are suitable substrates for the production of simple sugars.

Further work is in progress to establish the recyclability of the immobilized enzymes in the hydrolysis of pretreated Sila sorghum stalks in order to achieve cost reductions through recycling.

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