Enzymatic Hydrolysis of Blend of Lignicellulosic Materials for Reducing Sugar Production: Screening of Significant Process Factors

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The screening of the process parameters on the enzymatic hydrolysis of a blend of two lignocellulosic materials - corn cob (CC) and deseeded fluted pumpkin fruit (DFPF) using *Trichoderma reesei* was the focus of the present study. Four process parameters – time, temperature, substrate blend ratio and pH were screened for statistical significance using a 4 x 4 matrix of Graeco-Latin square design of experiment. The reducing sugar yield was determined using the dinitrosalisylic acid (DNS) method and maximum reducing sugar yield of 57.92mg/ml obtained in 2days at 40°C, blend ratio of 0.2:0.8 (CC:DFPF) and pH 5.5. Result of the Analysis of Variance (ANOVA) obtained showed that with 3 degrees of freedom and 6 residual degree of freedom at 95% confidence level (i.e. 0.05 significance), time, temperature and substrate blend ratio significantly affected the enzymatic hydrolysis process whereas the effect of pH was not significant. However, only time was significant at 99% confidence level (0.01 significance) while temperature, substrate blend ratio and pH were not significant. This study has highlighted the significant factors among the process variables in emzymatic hydrolysis of a blend of CC and DFPF.

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1. INTRODUCION

Bioethanol production has remained one of the key subjects in the production of renewable fuel with its advantage over fossil-derived ethanol lying in its renewability since the raw materials are of biomass origin. Literature reports that the sources of fossil fuel have been reducing significantly [1]. Production of bioethanol passes through three major processes: (1) a pre-treatment process involving either chemical or physical process which makes the biomas amenable to hydrolysis; (2) substrate hydrolysis (which could be enzyme or chemical catalysed) to convert cellulose or starch into reducing sugar and (3) fermentation process (enzyme catalysed) which converts the reducing sugar into bioethanol [2]. There is an increased interest in alternative fuels, especially liquid transportation fuels [3] and bioethanol is one of the most employed liquid biofuels due to its easy adaptability to existing engines and equally because it is a cleaner fuel with higher octane rating than gasoline [4]. One of the major challenges in bioethanol production is that it affects food security when produced from food stuff such as sugar cane, corn, sorghum, cassava, sugar beet etc. According to Ort and McMahan [5], saccharification of these food materials have resulted in good reducing sugar yield which consequently resulted in high bioethanol yield. The economic realities of the present time have caused a surge in demand for food and other essential materials such as ethanol. The cost of corn and other edible renewable feedstock has also been increasing as a result of the high demand for ethanol production [6]. According to 2020 Global Report on Food Crises, an estimated 135 million people are already in food crisis and suffering acute hunger in 55 countries and territories analysed. These, among other factors, cause a push for further research on improving the yield of bioethanol from second generation feedstock. These second generation feedstock comprising mainly of lignocellulosic materials have been reported to potentially contain large amount of energy and have the potential for bioethanol production [7] while equally offering the advantage of less competition to food [8]. However, large quantities of these wastes are underutilised in Nigeria [9]. The enzymatic hydrolysis of lignocellulosic biomass is one of the obstacles in the process of sugar production for bioethanol due to the presence of lignin that protects the cellulose molecules against cellulases [10] thereby leading to notorious resistance of cellulose to hydrolysis [11]. Ye and Berson [12] reported that enzymatic conversion of cellulose substrate is slow and presents one of the key bottlenecks that hamper the industrial development of ethanol from biomass. Usually, the yield is low. Many factors such as temperature, pH, mixing, substrate blend ratio, time, enzyme dose etc have been reported to affect the enzymatic hydrolysis process of these lignocellulosic materials [13-15]. Hence, a good understanding of the key process parameters from beginning through screening is desirable so that they can be more appropriately engineered in subsequent experiments for improved reducing sugar yield and consequently improved bioethanol yield.

Screening is the process of discovering through statistical design of experiment and modelling, those controllable factors or input variables that have a substantive impact on the response or output which is either calculated from a numerical model or observed from a physical process [16]. It does not have to be expensive and interest is not in the interaction among process parameters. This is because the aim is to study as many factors as possible in a minimum number of trials (runs) and to identify those that need to be studied in further rounds of experimentation in which the interactions can be more thoroughly assessed [17]. It is important to identify the factors that play important roles in the enzymatic hydrolysis of the blend of these lignocellulosic materials. By so doing, the not-too-important (non-significant) factors could be dropped and attention given to the important (significant) factors in further optimisation study. This constitutes the objectives of the screening experiment.

A Graeco-Latin Square design is a design of experiment in which the experimental units are grouped in three different ways and is obtained by superimposing two Latin squares of same size such that if every Latin letter coincides exactly once with a Greek letter, the two Latin Square designs are orthogonal and, together, they form a Graeco-Latin Square design. In this design, each treatment (Latin letter) appears just once in each column and once with each Greek letter [18]. In constructing Graeco-Latin squares,

numbers are frequently used instead of Greek characters [19].

Hence, identifying the factors that significantly affect the enzymatic hydrolysis of these substrates is very necessary as it will help in facilitating the optimisation process in subsequent study for improved reducing sugar yield as optimisation will be based on only the significant factors. It will also reduce the cost/number of runs during the optimisation experiment as attention would be given only to the factors that are important. It is always helpful and crucial when looked at from the view point of economics. Okpe et al. [20] used Graeco-Latin square to screen the factors that affect adsorption of Orange-G dye before optimizing only the significant factors.

In this study, corn (*Zea mays*) cob was blended with deseeded fluted pumpkin (*Telfairia occidentalis*) fruit and the blends subjected to enzymatic hydrolysis with the aim of screening for (to determine) the key factors that significantly affect the enzymatic hydrolysis process.

2. MATERIALS AND METHODS

2.1 Sample Preparation and Pretreatment

Lignocellulosic materials used in this research are corn cob (CC) and deseeded fluted pumpkin fruit (DFPF) sourced from agro waste dump in South-East Nigeria. Each of the collected samples was washed with clean water to remove dirt, sun-dried, underwent size reduction using hammer crusher, pulverized to fine particle size, pretreated with 1% NaOH for 2 hours to enhance enzymatic hydrolysis and then oven-dried to a constant weight at 102°C. The treated samples were separately stored in cellophane bags for use. The microorganism used in this work is *Trichoderma reesei* isolated from the dump site.

2.1.1 Procedure for determining the composition of corn cob and deseeded fluted pumpkin

2.1.1.1 Cellulose

The cellulose content of the samples was determined using Kurscher-Hoffer's method as adopted by Borysiak [21]. 10 g of biomass was heated in ethanol-nitric acid mixture under reflux for 1hr. After the first cycle, the liquid was decanted and the biomass flooded with fresh ethanol-nitric acid mixture again and heated under reflux for another 1hr cycle. The precipitate

was then washed with hot water and, then, flooded with hot water and heated under reflux for 30min. At the end of the third cycle, the precipitate was filtered off and the remnant washed with distilled water until neutral pH was achieved. It was air-dried and, then, oven-dried to a constant weight at 105°C. The difference in weight of the biomass before and after the process gave the cellulose content in %(w/w).

2.1.1.2 Extractive

3g of dried biomass was heated with the aid of a soxhlet extractor using acetone as solvent for extraction at a constant temperature of 90°C for 4hr and residence time for boiling and rising 70 and 25min respectively. The extractive-free sample(residue) was air-dried before being oven-dried for 1hr at 105°C to a constant weight, allowed to cool in a desiccator and weighed. The difference in weight of the sample (before and after extraction) was expressed as the %(w/w) of the extractive content [22].

2.1.1.3 Hemicellulose

The hemicellulose content of the biomass was determined using the method adopted by Amoah et al. [23]. 150ml NaOH solution was added to the residue from the extractive analysis and the mixture boiled for 3.5hr with recycled distilled water after which the residue was filtered and washed very well to remove sodium ion. The residue was then air-dried, oven-dried to a constant weight at 105°C, cooled to room temperature in a desiccator and weighed. The hemicellulose content was expressed in %(w/w) in equation (1)

$$W(wt\%) = \frac{G_1 - G_2}{G_O} \times \frac{100}{1} \qquad (1)$$

Where G_1 = weight of residue from extractive

G_2 = weight of residue dried in desiccator after NaOH treatment

G_o = weight of dry biomass before extraction

2.1.1.4 Lignin

The lignin content was determined using NREL lab procedures as adopted by Ayeni et al. [22]. 3ml of 72% H_2SO_4 was added to 0.3g of extractive-free biomass in a test tube at 30°C with careful shaking for 2hr at 30min interval to enable proper mixing and complete hydrolysis. 84 ml of distilled water was added to the system

at the end of the initial hydrolysis. The content was autoclaved at 121°C for the second step of hydrolysis for a period of 1hr after which the slurry was allowed to cool at room temperature. The obtained hydrolysate was subjected to vacuum filtration using a filtering crucible. The fraction of acid soluble lignin (ASL) was determined by measuring the absorbance of the hydrolysate at 320nm. To determine the acid insoluble lignin (AIL), the hydrolysed sample was oven-dried at 105°C, weighed and incinerated at 575°C to a constant weight in a muffle furnace. The incineration was to account for ash in determining the acid insoluble lignin. The lignin content is the summation of the acid soluble lignin and acid insoluble lignin expressed in wt%.

2.1.1.5 Ash

Ash content of the biomass was determined using a muffle furnace [24]. 20g of biomass was put in a porcelain crucible and heated in a muffle furnace at 575°C for 5hr. The crucible was removed and put in a desiccator and allowed to cool to room temperature. The process was repeated again and again, each time for 2hr, until a constant mass was obtained and the ash content (%w/w) was calculated on oven-dry basis as given in equation (2).

$$Ash\ (wt\%) = \frac{M_{Ash}}{M_{dry}} \times \frac{100}{1} \qquad (2)$$

Where M_{ash} = mass of ashed sample
M_{dry} = original mass of dry biomass

2.2 Enzymatic Hydrolysis Process

The enzymatic hydrolysis was carried out in 250cm^{3} conical flask containing 5%(w/v) inoculum and 5g of pretreated substrate (corn cobs and deseeded fluted pumpkin fruits blend) and incubated on a shaker at 150rpm at varying temperature, time, substrate blend ratio and pH ranging from 30-60°C, 1-5days, 0.2-0.8(w/w) and pH 1.5-7.5 respectively. The reducing sugar yield was determined using the dinitrosalisylic acid (DNS) method as adopted by Saliu and Sani [25]. This is an analytical technique for quantitative determination of the concentration of reducing sugar and is based on the detection of free carbonyl C=O group of reducing sugars in a given sample. In this work, 1ml of DNS solution was added to 3ml of the CC-DFPF hydrolysate and the mixture heated in a water bath at 100°C for about 10min until a red-brown colour developed. 1ml of sodium sulphate solution was added to stabilise the colour and the absorbance of the medium read at 540 nm [26] and the corresponding concentration determined using glucose calibration curve. The Graeco-latin square design for the screening of significant factors is given in Table 1.

Table 1 presents the 4 X 4 matrix Graeco-Latin Square design used in this experiment. It has a total of 16 runs. Where,

A-D=Time
T_I-T_4= Temperature
M_1-M_4=Substrate blend ratio
and 1-4=pH

2.2.1 Preparation of glucose calibration curve

200mg/ml glucose stock solution was prepared by dissolving 20g of analytical glucose in distilled water and making the volume up to 100ml. The stock solution was used to prepare different dilutions of the standard solution as shown below in Table 2 and the obtained data plotted in Fig. 1.

3. RESULTS AND DISCUSSION

3.1 Substrate Proximate Composition before and after Alkali Pretreatment

The result for the substrates characterisation before and after pretreatment was shown in Table 3. It showed the positive impact of alkali pretreatment process on both corn cob and deseeded fluted pumpkin fruit as their cellulose contents increased from 44.24% and 32.08% to 51.67% and 36.75%, with consequent decrease in lignin contents from 17.61% and 15.27% to 16.21% and 13.48% respectively. The result agreed with the report by Satari et al. [8] that NaOH is capable of reorganising the hydrogen bond network structure of cellulose thereby decreasing cellulose crystallinity and thus facilitates glucan digestibility. The table equally showed observable decrease in their hemicellulose content from 31.91% to 25.88% for corn cob, and 36.92% to 34.06% for deseeded fluted pumpkin fruit. This is equally in line with the report by Alvarez et al. [27] that pretreatment affects the hemicellulose composition and lignin structure of lignocellulosic materials. However, the pretreatment process had little effect (which could be considered negligible) on the extractive and ash content of the samples as can be seen from the table.

3.1.1 Result for enzymatic hydrolysis factor screening

The result of the enzymatic hydrolysis of blends of corn cob and deseeded fluted pumpkin fruit was presented in Table 4. A look at the result showed the reducing sugar yield ranging from minimum value of 18.35 mg/ml obtained at 60°C, 1day, pH 5.5 and blend ratio of 0.8:0.2(w:w) to a maximum reducing sugar concentration of 57.92mg/ml obtained at 40°C, 2days, pH 5.5 and blend ratio of 0.2:0.8(w:w). However, the result as it appeared did not quantify the effects of each of the factors being studied on the process. Hence, analysis of this result was carried out using analysis of variance (ANOVA) as shown in Table 5.

Table 1. Graeco latin square design of experiment for screening of factors for enzymatic hydrolysis

X_2 \ X_1	T_1	T_2	T_{352}	T_4
M_1	A1	B3	C4	D2
M_2	B2	A4	D3	CI
M_3	C3	D1	A2	B4
M_4	D4	C2	B1	A3

Table 2. Table for glucose calibration curve

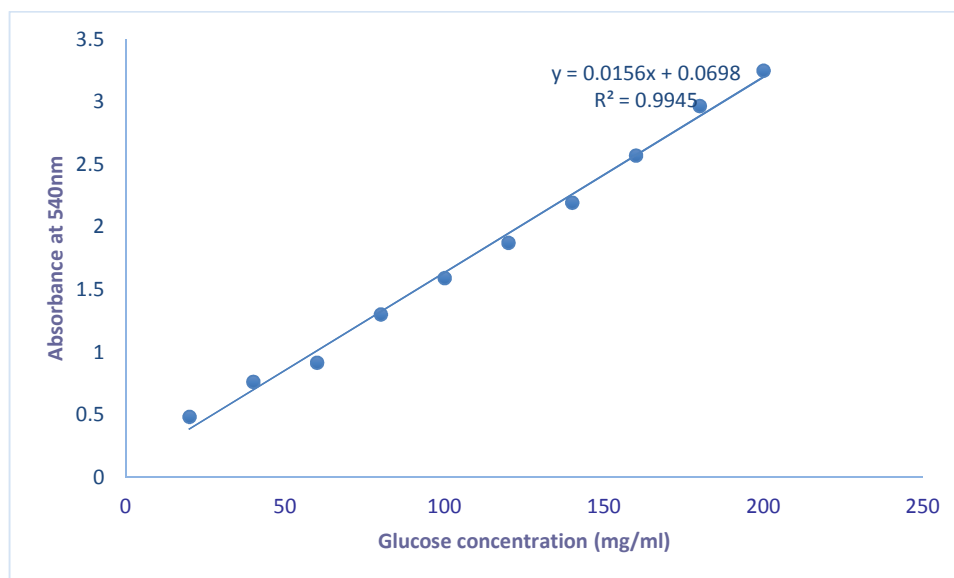| S/N	Volume of stock solution used (ml)	Final volume of solution (ml)	Glucose Conc. (mg/ml)	Absorbance at 540nm
1	10.0	10.0	200.0	3.250
2	9.0	10.0	180.0	3.011
3	8.0	10.0	160.0	2.751
4	7.0	10.0	140.0	1.994
5	6.0	10.0	120.0	1.673
6	5.0	10.0	100.0	1.391
7	4.0	10.0	80.0	1.300
8	3.0	10.0	60.0	0.914
9	2.0	10.0	40.0	0.692
10	1.0	10.0	20.0	0.481

Fig. 1. Glucose calibration graph

Table 3. Lignocellulose characterisation of samples (%w/w) before and after pretreatment

Sample	Cellulose		Hemicellulose		Lignin		Extractives		Ash	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
CC	44.24+0.12	51.67+0.23	31.91+0.17	25.88+0.13	17.61+0.22	16.21+0.14	2.75+0.09	2.76+0.07	3.49+0.08	3.48+0.08
DFPF	32.08+0.18	36.75+0.26	36.92+0.07	34.06+0.13	15.27+0.18	13.48+0.14	9.18+0.09	9.16+0.09	6.55+0.07	6.55+0.06

Table 4. Graeco Latin square design with response for screening of factors for enzymatic hydrolysis

Run	Temp (^oC) T_1-T_4	Time (day) A-D	Substrate blend ratio (w:w) M_1-M_4	Ph 1-4	Glucose Conc. (mg/ml)
1	30	1	0.2:0.8	1.5	29.90
2	40	2	0.2:0.8	5.5	57.92
3	50	3	0.2:0.8	7.5	33.72
4	60	4	0.2:0.8	3.5	43.61
5	30	2	0.4:0.6	3.5	41.22
6	40	1	0.4:0.6	7.5	34.39
7	50	4	0.4:0.6	5.5	53.68
8	60	3	0.4:0.6	1.5	18.73
9	30	3	0.6:0.4	5.5	42.02
10	40	4	0.6:0.4	1.5	44.69
11	50	1	0.6:0.4	3.5	39.79
12	60	2	0.6:0.4	7.5	46.50
13	30	4	0.8:0.2	7.5	46.03
14	40	3	0.8:0.2	3.5	53.88
15	50	2	0.8:0.2	1.5	47.05
16	60	1	0.8:0.2	5.5	18.35
	X_2	X_3	X_1	X_4	

3.1.2 Analysis of variance (ANOVA)

The reducing sugar yield from the enzymatic hydrolysis of CC-DFPF blends was presented in Table 4 and the statistical analysis (ANOVA) of the result was presented in Table 5 which quantified the relative effects of the factors being studied on the enzymatic process [28]. The ANOVA aids in analysis and validating the experiment result of any process [29]. The ANOVA table showed that at 5% confidence level X_1, X_2 and X_3 were all significant while X_4 was not significant because its variance ratio, F_4 was less than $F_{0.05}(3,6)$ from F-distribution table [19]. This implied that, substrate blend ratio, temperature and time were significant factors that affected the enzymatic hydrolysis of a blend of corn cob and deseeded fluted pumpkin fruit using *T.reesei*. However, the effect of pH was not significant at that confidence level. This means that increase/decrease in pH in the reaction will not have much effect on the yield of glucose. The result partly agrees with Sen [30] who studied the factors affecting enzymatic hydrolysis of corn cob using a mathematical tool, Principal Component Analysis (PCA) to underline the key factors that determine the enzymatic hydrolysis of pretreated lignocellulosic biomass (corn cob) in which the results of PCA indicated that enzyme reaction pH 5.5, and incubation temperature of 45 °C were suitable for high concentration of glucose. However, the order in which the parameters affected the enzymatic hydrolysis process was not stated. It was also in reasonable agreement

with the report by Fenila and Shastri [13] that temperature has significant impact on the enzymatic hydrolysis of lignocellulosic materials. The statistical significance of substrate blend ratio obtained in this work corroborates the report by Oke et al. [14] that if mixed lignocellulosic biomass feedstock components are used in the appropriate ratios that optimal yield of fermentable sugars which would result in higher bioethanol yield could be obtained. This is in close agreement with Yen and Berson [12] whose work on factors affecting cellulose hydrolysis based on inactivation of adsorbed enzymes reported that increasing reaction temperature would cause a significant increase in the inactivation rate in addition to the catalytic reaction rate.

At 99% confidence level, $F_{0.01}(3,6) = 9.78$ from the table. Hence, only time is the factor that significantly affected the enzymatic hydrolysis at that significance level. This is justified by the high value of F_3=15.406 which is greater than $F_{0.01}(3,6)$=9.78, whereas F_1,F_2 and F_4 are all less than F-value from table at 0.01 significance level. This means that the effect of substrate blend ratio, temperature and pH are all not significant at this level. This is to say that the effect of time will mostly affect the yield of glucose in the enzymatic hydrolysis of a blend of corn cob and deseeded fluted pumpkin fruit, followed by temperature and lastly by substrate blend ratio, while the effect of pH will not drastically affect the yield of reducing sugar (glucose) during the

Table 5. ANOVA table for screening of factors for enzymatic hydrolysis

Source of variance	Sum of squares	No of degrees of freedom	Estimate of variance	Variance ratio
X_1-Substrate Blend ratio	264.99	3	88.33	7.54
X_2-Temperature	303.650	3	101.217	8.64
X_3-Time	541.46	3	180.487	15.406
X_4-pH	137.67	3	45.89	3.92
Residual	70.288	6	11.715	
Total	1320.01	15		

$F_{0.05}(3,6) =4.76$

enzymatic hydrolysis of a blend of corn cob and deseeded fluted pumpkin fruits.

It should be noted that even though time, temperature and substrate blend ratio were determined to be significant in this study, it does not mean that pH does not have effect. The implication is that even though pH has effect on the enzymatic hydrolysis process, its effect in the reaction/process can be neglected in subsequent experimentation since either increasing or decreasing it has very little effect on the system, while neglecting any of time, temperature or substrate blend ratio would cause a great increase or decrease in the yield of reducing sugar. This is in reasonable agreement with Egbuna et al. [31] that non-significant factors can be kept constant in subsequent study.

4. CONCLUSION

Factors affecting the enzymatic hydrolysis of lignocellulosic biomass were successfully screened using Graceo-Latin square. The factors were time, temperature, pH and substrate blend ratio while the lignocellulosic biomass was a blend of corn cob and deseeded fluted pumpkin fruits. The substrate proximate analysis indicated that the biomass contains cellulose and hemicelluloses in significant quantities. The analysis of variances showed that at 95% significance level, time, temperature and substrate blend ratio significantly affected the enzymatic hydrolysis process while the effect of pH was not significant. Hence a little manipulation of any of them would either increase or decrease the yield of reducing sugar significantly in the enzymatic hydrolysis of a blend of corn cob and deseeded fluted pumpkin fruit. Hence, optimization of process variables in the enzymatic hydrolysis of these lignocellulosic materials should focus on determining the optimum conditions of these independent factors for optimal reducing sugar yield and consequently optimal ethanol yield.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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