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OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF BANANA PEELS FOR BIOETHANOL PRODUCTION

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ABSTRACT

This research work investigated the Optimization of the enzymatic hydrolysis of banana peels for bio-ethanol production. Aspergillus niger was isolated and identified from rotten cassava tubers and subsequently maintained on potato dextrose agar (PDA) slants. A crude enzyme was prepared from the isolated enzyme and used for the hydrolysis of banana peels. The characterization of banana peels was conducted using Fourier Transform Infrared Spectroscopy (FTIR) and proximate analysis. The yields of reducing sugar and ethanol from hydrolysis were optimized using Response Surface Method (RSM) via the Central Composite Design (CCD). The effects of incubation temperature on the ethanol yield from banana peels revealed that low temperature favoured ethanol yield, while high temperature and high pH are unfavourable for maximum ethanol production. The optimum condition for banana hydrolysis was observed to be temperature: 35 ^{o}C, pH: 5.5, and hydrolysis period: 5.7 (days).

Keywords: Cellulose, Hemicelluloses, Aspergillus Niger, Central Composite Design (CCD), Biomass, Inoculum.

1. INTRODUCTION

Ethanol has gained recognition as a potential fuel source due to the continuous rise in the price of conventional fossil fuels, the uncertainty of the availability of petroleum resources to meet the fuel needs of a growing population, and concerns about global climate change [7].

Due to the abundant availability of agricultural residues and the renewable nature of these substrates, ethanol production from such substrates appears to have immense commercial potential. Use of ethanol as a partial replacement for gasoline because of its important characteristics, such as high octane number and the ability to burn clean, resulting in reduced CO emission, has been well documented [4] [7].

The economics of bioethanol production by fermentation is significantly influenced by the process and cost of raw materials, which account for more than half of the production cost [12] Feedstock flexibility is also important for the successful commercial ethanol production for its use as a biofuel [7]. Different biofuels such as ethanol, methanol, bio-diesels, etc. are produced by fermentation of agricultural wastes, fruit wastes, municipal and industrial wastes through the process of enzymatic hydrolysis. This process of breaking down plant biomass (enzymatic hydrolysis), such as cellulose, into sugar, which is then converted into bioethanol, is a vital factor in the production process and thus determines the viability of the entire process. Amongst these biofuels so produced, ethanol has the greatest demand because of its desirable properties, such as clean burning, among others [16] [1].

Several metric Millions of biomass waste in the form of banana peels are deposited each year in Nigeria. These wastes from food processing industries, farm products spoils, and household-generated waste constitute a nuisance to the environment when not properly disposed [13]. Utilization of these bio-wastes in the production of ethanol will help solve the problems of diversification, waste disposal, and energy needs.

2. MATERIALS AND METHODOLOGY

2.1 Raw material sourcing

Fresh peels of banana were obtained from a local market in Mgbakwu, Awka, in Anambra State. The samples were identified as Musa sapientum (banana) in the Department of Botany, Nnamdi Azikiwe University, Awka.

2.2 Sample preparation:

The banana peels were washed and dried in a cabinet oven at 50 ^{0}C for 24 hours. The dried sample was then milled into fine flour using a milling machine, sieved, and packaged in air-tight polyethylene bags and labelled for analysis

2.3 Characterization of the Banana peel

Fourier Transform Infrared Spectroscopy (FTIR) of banana peels was carried out to determine the functional groups. A Shimadzu Analytical FTIR-8400S was used.

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2.4 Determination of the proximate composition of the peel

The crude protein was analyzed using the Kjedal method, and the fats and oils were measured using the Sohxlet method [18]. The lignin and ash contents of each peel sample were measured by treating the peels with 72% w/w H2SO4 for 4 hrs. The suspension after treatment with the acid was filtered through a crucible, and the solid residue was dried at 105˚C for 24 hours and weighed (W1). The residues were then transferred to a pre-weighed dry porcelain crucible and heated at 600˚C for 6 hours. After cooling down, it was weighed (W2) as the ash content. The lignin was calculated by the difference (W1-W2) [18].

To determine the concentration of cellulose/hemicelluloses in the banana peels, the method of Kurschner-Hanack [18] [9] was used.

2.5 Crude enzyme preparation and concentration of the enzyme

Cultures were dispensed into 100ml conical flasks and incubated at 30^0C with rotary shaking at 100rpm for 72hours, after which mycelia pellets were separated by filtration through glass wool at 30^0C. The resultant cell-free filtrate was used as a crude enzyme. The crude enzyme filtrate (250ml) was concentrated by dialysis in a 4M sucrose solution overnight. The dialysate was screened for cellulose activities and further dialyzed in 6M sucrose solution for 24 hours. [18].

2.6 Determination of cellulose activity in crude enzyme

The enzyme activity was determined using the methods of Wang et al. (1988). The reaction mixture consisted of 0.5ml of 0.05g of carboxymethylcellulose in 100ml 0f 0.2M phosphate buffer (pH 7) and 0.5ml of crude enzyme solution. This substrate-enzyme mixture was then incubated at 40 ^{0}C for 30 min. in a water bath. The reaction was stopped by adding 1ml of 3, 5– 3,5-dinitrosalicylic acid (DNS) to the reaction mixture. After, the mixture was heated in boiling water for 10 minutes and cooled under running water. Four millilitres (4ml) of distilled water were added to the mixture and the absorbance determined at 540nm using a spectrophotometer. One unit of cellulose was defined as the amount of enzyme that liberated 1µmol of reducing sugar per minute under assay conditions.

2.7 Hydrolysis inoculums

The inoculum (1%) of the multiplied A. niger from a PDA slant was prepared by aseptically transferring 10 g of the pure and screened A. niger from the slant to a 1-liter volumetric flask. Distilled water autoclaved at 121˚C for 15 mins was added to mark the flask. The mixture was left for 5 to 10 minutes at 150 rpm. The inoculum size was set to have a cell concentration of 1.0×10^8 cells per ml [3] [9]. The whole of the hydrolysis experiment was carried out using this inoculum.

2.8 Enzymatic hydrolysis with Aspergillus niger

The enzymatic hydrolysis was carried out at different temperatures, for different time intervals, and at different pH levels. This was done using a 250 cm^3 conical flask containing 50 cm^3 of 5% inoculum of A. niger with different dosages of the peels was used to carry out the enzymatic hydrolysis. The mixture was incubated on a shaker with an agitation rate of 300 rpm and subsequently filtered. The soluble sugar yield in the filtrate was measured using the refractometer, while the reducible sugar yield was determined using the DNS method [11] [9].

2.9 Fermentation of the hydrolyzed banana peels

A 250 cm^3 conical flask containing 30cm^3 of medium obtained from the enzymatic hydrolysis (Ahmad et al., 2011) was used to carry out the fermentation. The medium was inoculated with 5% (v/v) growth medium containing the activated Saccharomyces cerevisiae and incubated on a shaker with an agitation rate of 300 rpm at 30˚C for 5 days at pH of 4.5 [10].

2.10 Distillation of the ethanol produced

A simple distillation setup was used to carry out the distillation of the ethanol produced. The liquid fermented was transferred into round round-bottom flask. The round-bottom flask was then placed on a heating mantle fixed to a distillation column enclosed in running tap water. A second flask was fixed to the other end of the distillation column to collect the distillate at 78˚C [14].

2.11 Determination of ethanol quantity

The distillate collected was measured using a measuring cylinder and expressed as the quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol [14]. The concentration of ethanol was determined by using a specific gravity meter (which can measure the ethanol percentage directly) or by comparing the density of the ethanol produced with the standard ethanol density curve [8].

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3. MODELING AND ANALYSIS

3.1 Optimization of the hydrolysis process

The optimization studies on the hydrolysis process were carried out using a Central Composite Design (CCD) of the Response Surface Methodology. This was studied to determine the optimum reducing sugar and ethanol yield and the best conditions or optimum levels of the factors for the optimum reducing sugar and ethanol yield. The interactive effects of the three important factors (which are temperature, pH, and time) on reducing sugar and ethanol yield from banana and cocoyam peels, respectively, were investigated. The reducing sugar yield and ethanol yield are the dependent variables or responses for hydrolysis and fermentation, respectively, while the three important factors are the independent variables. The design matrices for the hydrolysis and fermentation process of banana peels optimization are presented in Tables 3.1 and 3.2, respectively.

Table 3.1: Upper and lower limits for the optimization of the hydrolysis process

Parameter	Lower limit	Upper limit
Temp. (°C)	30	40
pH	3.5	7.5
Time (day)	1	9

Table 3.2: Design matrix for the optimization of the hydrolysis process

	Factor 1	Factor 2	Factor 3
Run	**X_1:Temp.(°C)**	**X_2:pH**	**X_3:Time (Day)**
1	40	7.5	9
2	35	5.5	5
3	35	5.5	5
4	35	5.5	1
5	40	5.5	5
6	30	3.5	9
7	35	5.5	5
8	30	5.5	5
9	40	3.5	9
10	30	3.5	1
11	35	7.5	5
12	40	3.5	1
13	35	5.5	9
14	40	7.5	1
15	35	5.5	5
16	35	5.5	5
17	30	7.5	1
18	35	3.5	5
19	30	7.5	9
20	35	5.5	5

3.2 Analysis of variance (ANOVA)

Analysis of Variance (ANOVA) is a statistical test used in the determination of the equality of more than two population means. Test statistics (F) use the F-distribution (probability distribution) function and information about the variances of each population and grouping of populations to help decide if the variability between and within each population is significantly different. The test statistic was computed using equation 1 [5].

$$\textbf{Test statistic (F)} = \frac{\textbf{Treatment Mean Square (TMS)}}{\textbf{Error Mean Square (EMS)}} \quad (1)$$

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But Error mean square which is one of many ways to quantify the difference between values implied by an estimator and the true values of the quantity being estimated, was calculated using equation 2.

$$\textbf{Error mean square (EMS)} = \frac{\textbf{Error sum of squares (SSE)}}{\textbf{Error degree of freedom (df)}} \quad (2)$$

SSE Total Sum of Squares (SS) - Treatment Sum of Squares (SST)

Where SSE is the error sum of squares

$$\textbf{Treatment mean square (TMS)} = \frac{\textbf{Treatment sum of squares (SST)}}{\textbf{Degree of freedom (df)}} \quad (3)$$

Sum of squares for treatments (SST) = (Sum of squares of treatments totals with each square divided by the number of observations for that treatment) – Correction for mean.

3.3 Degree of freedom

Since each sample has degrees of freedom equal to one less than their sample sizes, then treatment and error degrees of freedom were calculated using equations 4 and 5, respectively [5].

Degree of freedom (df) = T-1 (4)

Error df = T(r -1)

4. RESULTS AND DISCUSSION

4.1 Characterization of the banana peel

The results from the proximate analysis of the banana peels are presented in Table 4.1. From the result, it could be observed that the banana peels have a lignin content of 7.8% which is in agreement with the lignin content value of 6 – 12% reported in the literature [2][15]. The value of ash content for the banana peels is lower than the value of 8.5% published in the literature [2] [15]. While the moisture content of the banana peels is consistent with the value of 6.7% reported by Hiren et al. [19]

Table 4.1: Proximate composition of banana peels (%)

Parameters	Samples (%)
	Banana peels
Protein	6.0±0.10
CHOs	20.30±0.10
Fibre	20.40±0.10
Fats	<6.20
Tannins	1.52±0.10
Moisture content	8.05±0.10
Sugar	30.05±0.10
Lignin	7.80±0.10
Ash	0.50±0.10

4.2 FTIR studies of banana peels and cocoyam peels

The results of the FTIR studies for the banana peel are presented in Figure 4.1. The results were analyzed based on the standard peaks presented by Silvestein et al. (1981) for various functional groups and their interpretation presented in Table 4.2

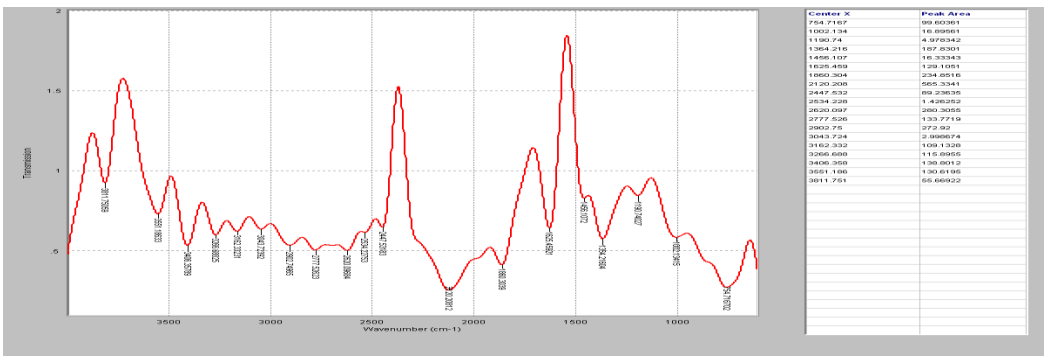

Figure 4.1: FTIR result of banana peels

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It can be seen that the banana peels contain OH group for Alcohols, Phenols, acids and carboxylic acids, which are essential functional groups in cellulose materials. Other functional groups, like amines, amides, aldehydes, esters, ethers, alkenes, and di-substituted benzenes, were also observed to be present

Table 4.2: FTIR Interpretation table for Banana peels

Wave number	Peak Intensity	Functional group
3551.186	Strong, Weak	O-H (free) for Alcohols, Phenols
3406.358 3266.688	Medium	NH or O-H (H-bonded) for Amines, Alcohols, phenols
3043.724	Sharp, Strong	C-H Stretch for Alkenes, Arenes
2902.75	Very broad	O-H for Acids, Carboxylic acids
2777.526	Medium	CHO for Aldehydes
1625.459	Medium	C=C Stretch for Alkenes
1456.107	Variable	C=c for Arenes
1190.74	Strong	C-O for Acids, esters
754.7167	Strong	1,3-di-substituted benzene

4.3 Concentration of enzymes

This was observed to have a reduced protein content of 3.60 mg/ml from 12.0 mg/ml and an increased specific activity of 1.94 µ/mg from 1.75 µ/mg when concentrated. It was further dilated for another 24 hours, a volume of 32 ml and protein content of 1.54 mg/ml was obtained with the specific activity increased to 2.68 µ/mg. The complete result is presented in Table 4.3.

Table 4.3: Concentration summary of enzymes

Steps	Concentration Volume (ml)	Optical Density	Total activity (U/ml)	Total protein(mg/ml)	Spec.activity (µ/mg)
Crude enzyme	250	0.0360	21.00	12.00	1.75
Dialysis on:					
4M sucrose	75	0.450	6.98	3.60	1.94
Dialysis on:					
6M sucrose	32	0.0630	4.13	1.54	2.68

4.4 Banana peel hydrolysis: Statistical optimization

The design matrix and output response for the optimization of the hydrolysis process are presented in Table 4.4.

Table 4.4: Design matrix for the optimization of the hydrolysis of banana peels

	Factor 1	Factor 2	Factor 3	Response 1
Run	**X_1:Temp.(^oC)**	**X_2:pH**	**X_3:Time (Day)**	**Reducing sugar yield (mg/mol)**
1	40	7.5	9	118.1
2	35	5.5	5	121
3	35	5.5	5	121.1
4	35	5.5	1	119
5	40	5.5	5	119.6
6	30	3.5	9	119.8
7	35	5.5	5	121.2
8	30	5.5	5	120.3
9	40	3.5	9	119.3

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10	30	3.5	1	116.4
11	35	7.5	5	120.7
12	40	3.5	1	116
13	35	5.5	9	119.5
14	40	7.5	1	118.6
15	35	5.5	5	121.3
16	35	5.5	5	121.4
17	30	7.5	1	119.4
18	35	3.5	5	120.4
19	30	7.5	9	118.8
20	35	5.5	5	122

The summary of P-values indicates that a quadratic model fitted the ANOVA analysis, and hence, the quadratic models were suggested. A significance level of 95% was used; hence, all terms whose P-value is less than 0.05 are considered significant.

The ANOVA table is given in Table 4.5, at the significance level of 5%, all terms with P-values less than 0.05 are considered significant. From the ANOVA table, the regression F-value of 29.95 implies that the model is significant, which was validated by the P-value being less than 0.05. The P-values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables [17]. The larger the magnitude of the F-test value and the smaller the magnitude of the P-values, the higher the significance of the corresponding coefficient.

Table 4.5: ANOVA table for Banana peels hydrolysis

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	46.71623	9	5.190692	29.94735	< 0.0001	significant
X_1-Temp.	0.961	1	0.961	5.544425	0.0403	
X_2-pH	1.369	1	1.369	7.898353	0.0185	
X_3-Time	3.721	1	3.721	21.46806	0.0009	
X_1X_2	0.045	1	0.045	0.259624	0.0214	
X_1X_3	0.052	1	0.053	0.344533	0.0300	
X_2X_3	7.605	1	7.605	43.87653	< 0.0001	
X_1^2	2.434602	1	2.434602	14.04627	0.0038	
X_2^2	0.319602	1	0.319602	1.843924	0.02043	
X_3^2	7.404602	1	7.404602	42.72035	< 0.0001	
Residual	1.733273	10	0.173327			
Lack of Fit	1.099939	5	0.219988	1.736746	0.2797	Not significant
Pure Error	0.633333	5	0.126667			
Cor Total	48.4495	19				
Std. Dev.	0.416326			R-Squared		0.964225
Mean	119.695			Adj R-Squared		0.932028
C.V. %	0.347822			Pred R-Squared		0.853179
PRESS	7.11341			Adeq Precision		17.46619

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The Model F-value of 29.95 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

The "Pred R-Squared" of 0.8532 is in reasonable agreement with the "Adj R-Squared" of 0.9320. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 17.466 indicates an adequate signal. This model can be used to navigate the design space.

The initial quadratic model equation was obtained for the hydrolysis of banana peels for reducing sugar yield.

Reducing sugar yield(mg/mol) =

$$+65.50446 + 2.61380X_1 + 1.9943X_2 + 1.84838X_3 + 0.75003X_1X_2 + 0.03415X_1X_3 - 0.12187X_2X_3 - 0.037636X_1^2 - 0.085227X_2^2 - 0.10256X_3^2 \quad (6)$$

Final quadratic model equations obtained for reducing sugar yield optimization from banana peels hydrolysis, after eliminating the insignificant model terms, are expressed in equations (7).

Reducing sugar yield (mg/mol) =

$$+121.16 - 0.31X_1 + 0.37X_2 + 0.61X_3 - 0.075X_1X_2 + 0.04X_1X_3 - 0.97\ X_2X_3 - 0.94\ X_1^2 - 0.34\ X_2^2 - 1.64\ X_3^2 \quad (7)$$

The coefficient of regression R^2 was used to validate the fitness of the model equation. The R^2 has a high value of 0.964, showing that 96.4% of the variability in the response can be explained by the model. This implies that the prediction of experimental data is quite satisfactory.

The normal probability plot of residuals (Figure 4. 2) indicates that the residuals follow a normal distribution, in which case the points follow a straight line as indicated.

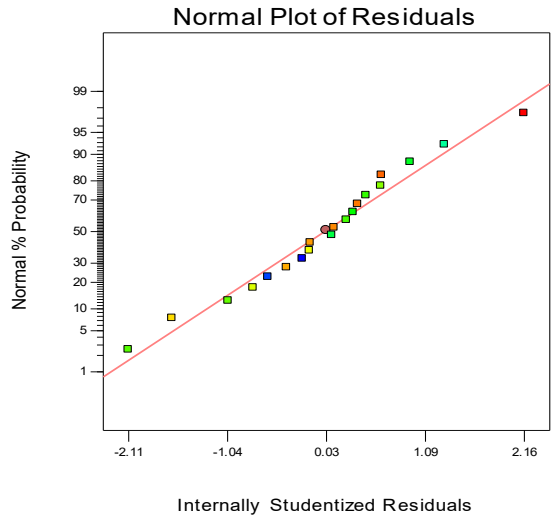

Fig 4.2: Normal Plots of Residuals from banana

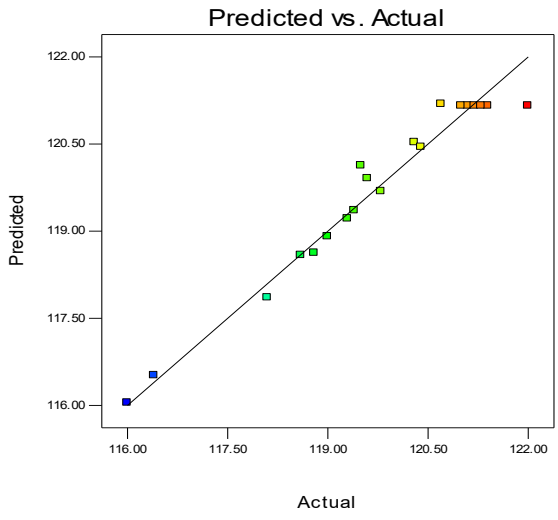

Fig 4.3: Plot of Predicted vs Actual from banana

A graph of the actual response values versus the predicted response values (Predicted vs Actual; Figure 4.3). These plots equally confirm that the selected model was adequate in predicting the response variables in the experimental values since it follows a straight line at 45 angle.

4.5 The three-dimensional (3-D) response surface plots for reducing sugar yield from banana peel hydrolysis

Figures 4.4 show that reducing sugar yield decreases with an increase in temperature. The maximum reducing sugar yield was achieved at a temperature of 35 °C. Figure 4.4 shows that the maximum yield of reducing sugar was achieved at a pH of 5.5. It could be observed that the yield of reducing sugar increased with an increase in pH up to pH 5.5, after which further increase in pH led to a decrease in reducing sugar yield. Figures 4.5 and 4.6 show that the yield of reducing sugar increased with an increase in fermentation time, after which further increase in the fermentation time led to a decrease in the yield of reducing sugar. The maximum reducing sugar yield was recorded against the fermentation period of 5.7 days. The optimum reducing sugar yield value of 121.2 (mg/mol) was recorded at the optimum fermentation conditions of temperature (35 °C), pH (5.5) and hydrolysis time (5.7 days).

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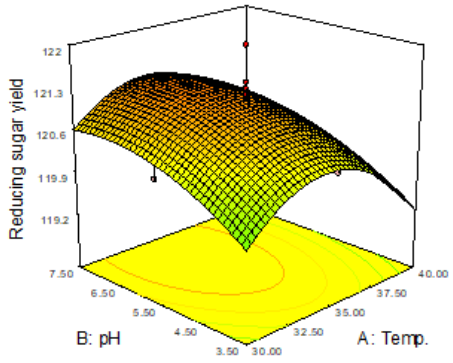

Fig 4.4: pH Vs temperature 3D plot for the hydrolysis

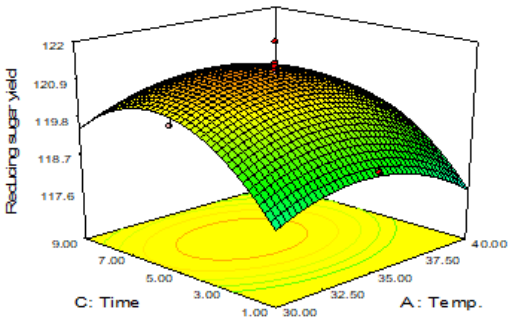

Fig 4.5: Time Vs temperature 3D plot for the hydrolysis

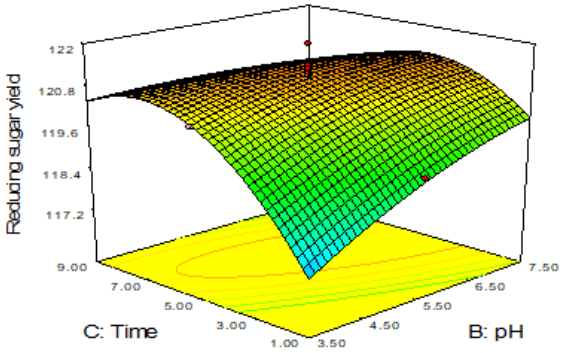

Fig 4.6: Time Vs pH 3D plot for the hydrolysis

4.6 Numerical optimization and validation of the optimization result.

The determination of the optimum levels of the hydrolysis process factors for maximizing reducing sugar yield is the primary objective of the optimization study. From Table 4.6, it can be seen that temperature: 35 (°C), pH: 5.5, and fermentation period: 5.7 (days) are the optimum hydrolysis process factors required for maximum reducing sugar yield. Under these hydrolysis process conditions, the predicted reducing sugar yield was 121.2 (mg/mol), which was in good agreement with the experimental value of 122 (mg/mol) performed at the same optimum values of the process variables. The optimization was performed using the numerical method of the Design Expert version 10.0 by State Ease U.S.A.

Table 4.6: The predicted optimum conditions and experimental validated result

Optimum Conditions Predicted					
Sample	**Temp. (°C)**	**pH**	**Time (day)**	**Predicted reducing sugar yield (mg/mol)**	**Experimental Validated Result (%)**
Banana peels	35	5.5	5.7	121.2	122

5. CONCLUSION

The characterization of the sample showed the presence of cellulose and OH functional groups for alcohol and phenols. This makes banana peel a suitable raw material for bioethanol production. Also, crude preparation of Aspergillus niger is effective in the hydrolysis of lignocellulosic materials to release reducing sugars. The quantity of reducing sugar and ethanol from the hydrolysis process depends on temperature, time, PH, and substrate concentration, while the yields increase with substrate concentration at low temperature. This indicates that high temperature and high pH are unfavorable for maximum yields in the bioethanol production process, where enzymatic hydrolysis is involved.

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