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OPTIMIZATION OF NUTRIENT MEDIUM FOR THE PRODUCTION OF BIOETHANOL FROM RICE HUSK USING *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

In this study, the composition of nutrients in the fermentation medium for the production of bioethanol from rice husk using Saccharomyces cerevisiae was optimised using a four variable central composite design (CCD) coupled with response surface methodology (RSM). The nutrient investigated were yeast extract, ammonium nitrate (NH_4NO_3), glucose and potassium dihydrogen phosphate (KH_2PO_4). The CCD was used to generate a statistical model for predicting the optimal levels of the nutrients. Results obtained from analysis of variance (ANOVA) showed that the model was statistically significant ($p<0.0001$) with a low standard deviation (0.097) and a high coefficient of determination ($R^2=0.985$). The optimum values of yeast extract, NH_4NO_3, glucose and KH_2PO_4 from RSM were 0.87 g/L, 0.16 g/L, 8.00 g/L and 0.03 g/L respectively. Under these conditions, the ethanol concentration was obtained as 3.41%v/v. Validation of the statistical model indicated no significant difference between experimental observations and model prediction. The observed results indicate the viability of rice husk as a biofuel feedstock and corroborates the efficiency of CCD in determining the accurate values of the nutrients where maximum production of ethanol occurs.

1. INTRODUCTION

Global depletion of fossil fuels, environmental concerns, and pressures for oil independence are factors that are creating a strong market for biofuels. Biofuels have the potential to be domestically and globally available for energy security, with most being carbon neutral,

potentially carbon negative and supportable within the current agricultural infrastructure (Isa et al., 2004). Bioethanol is one of the most important biofuels hence its dominance in the biofuel market (Amenaghawon et al., 2014). The main advantage of bioethanol is the possibility to blend it in low proportions with gasoline (5 to 25% bioethanol by volume) for use, without any significant change in internal combustion engines (Osunkoya and Okwudinka, 2011). Research has shown that bioethanol can be produced from sugar containing feedstocks (e.g. sugarcane, sugar beet), starch containing feedstocks (e.g. corn, barley, wheat) or lignocellulosic feedstocks (e.g. sugarcane bagasse, straw, wood and agro residues) (Cardona et. al 2007). The use of the first two types of feedstock which serves as the source of first generation of biofuels result in competition with food for consumption and in the long run can lead to food price hike (Amenaghawon et al., 2013). Lignocellulosic feedstock from which second generation biofuels are produced include agricultural and forest residues, municipal solid waste and energy crops. These have the potential to be an economical source of feedstock for bioethanol production as a result of their widespread availability, sustainable production, environmental benefits and low cost without endangering food security (Agbro and Ogie, 2012).

Rice husk is one of the most widely available agricultural wastes in many rice producing countries of the world. Rice husks are the hard protecting coverings of grains of rice which are removed from the rice seeds during the milling process. Rice husk consists of cellulose (32.24%), hemicellulose (21.34%), lignin (21.44%) and some mineral components such as silica, alkalis and trace elements (Rahman et al., 1997). Its unique chemical composition, renewability and abundant availability at little or no cost makes it a good candidate for producing bioethanol (Duku et al., 2011).

The production ethanol has been reported to be influenced by fermentation conditions such as type of microorganism, composition of fermentation medium, substrate type and concentration, agitation rate, aeration, temperature, pH etc (Asli 2010). However, ethanol production during fermentation is strongly influenced by the medium composition such as nitrogen, phosphorus, potassium and other salts. Thus optimum production of ethanol could be achieved by optimising the fermentation nutrient medium (Gopinadh et al., 2015). Optimisation of fermentation nutrient medium by the traditional one-factor-at-a-time method is often cumbersome and time consuming. Response surface methodology has been found to be very useful in optimising multivariable processes and it has been successfully applied to the optimisation of many bioprocesses (Amenaghawon et al., 2015; Gopinadh et al., 2015; Imandi et al., 2008; Thongdumyu et al., 2014).

The objective of this study was to optimize the production of bioethanol from rice husk via separate hydrolysis and fermentation using *Saccharomyces cerevisiae*. A four variable central composite design was used to study the effect of yeast extract, NH_4NO_3, glucose and KH_2PO_4 on the concentration of ethanol produced. Response surface methodology was used to obtain the optimum values of these variables as well as the chosen response (ethanol concentration). This was done by generating response surface plots showing the effect of any two variables on ethanol concentration while keeping the other two variables constant.

2. MATERIALS AND METHODS

2.1. Feedstock Collection and Pretreatment

Rice husk was obtained from a local mill in Minna, Niger State, Nigeria. The rice husk was milled using a standard laboratory mill and then sieved to obtain 1 mm particles. It was then washed with sterile water to remove any extraneous matter and dried in hot-air oven at 70°C to a constant weight. Delignification was carried out by soaking the biomass in 2M NaOH solution and autoclaving at 100°C for 1 hour. The resulting material was neutralized by continuous washing with warm distilled water until neutral pH was obtained. The delignified sample was oven dried at 60°C until constant weight (Agbodike et al., 2013).

2.2. Hydrolysis of Rice Husk

Acid hydrolysis of the delignified rice husk was carried out in 500 ml round bottom flask using dilute sulphuric acid of concentration 2.5 %w/w. The operating conditions of the hydrolysis reaction were as follows: temperature 100°C, time 30 minutes and liquid content of 250 ml acid. At the end of the hydrolysis reaction, the solid residue was separated by centrifugation and the hydrolysate was detoxified according to the method of Silva et al. (1998). The detoxified hydrolysate was stored for further use.

2.3. Culture Media and Fermentation

Fermentation was carried out in a 250 ml Erlenmeyer flasks using 100 ml of the hydrolysate. The pH of the hydrolysate was adjusted to 5.5 using 2M NaOH and 2M H_2SO_4 as desired. The fermentation medium had the following composition (per 100ml of hydrolysate): 0.1 g $FeNH_4(SO_4)_2$, 0.25 g $(NH_4)_2HPO_4$, 0.3 g Urea, 0.3g. Nitrogen and phosphorus supplementation was determined by the experimental design as follows NH_4NO_3 (0.05 to 0.5 g), yeast extract (0.5 to 1 g), KH_2PO_4 (0.01 to 0.1 g). The hydrolysate was also supplemented with glucose (2 to 10g) according to the experimental design. The flasks containing the medium was inoculated with 10 ml of activated dry Baker's yeast (*Saccharomyces Cerevisiae* 3 x 10^8 cells/ml) and fermentation was allowed to occur for a duration of 72 hours.

2.4. Analytical Methods

Liquid samples were taken from the fermentation broth at the end of fermentation. The samples were filtered using a Whatman's No 4 filter paper. The amount of ethanol produced was determined according to the method reported by Hadeel et al. (2013).

2.5. Experimental Design

A four variable central composite design (CCD) for response surface methodology was used to develop a statistical model for optimising the fermentation medium. The ranges of the variables that were optimized (yeast extract, NH_4NO_3, glucose and KH_2PO_4) are as shown in Table 1. Ethanol concentration was chosen as the response for process optimization using RSM. Experimental observations from the fermentation process was analysed using Design

Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA) and fitted according to Equation (1) as a second-order polynomial equation including main effects and interaction effects of each variable. Analysis of variance (ANOVA) and response surface plots were generated using Design Expert and the optimised value of the independent variables for optimum response was determined using numerical optimisation.

$$Y_i = b_o + \sum b_i X_j + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \quad (1)$$

Where Y_i is the dependent variable or predicted response, X_i and X_j are the independent variables, b_o is offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the error term.

Table 1: Experimental range and levels of independent variables

Independent Variable	Symbols	Coded and Actual Levels				
		-1.68	-1	0	+1	+1.68
Yeast Extract (g/l)	X_1	0.5	0.625	0.75	0.875	1
NH_4NO_3 (g/l)	X_2	0.05	0.1625	0.275	0.3875	0.5
Glucose (g/l)	X_3	2	4	6	8	10
KH_2PO_4 (g/l)	X_4	0.010	0.033	0.055	0.078	0.100

3. RESULTS AND DISCUSSION

3.1. Statistical Modeling

Analysis of the experimental data using the Design Expert software revealed that the quadratic model was significant for ethanol production. This was further confirmed by ANOVA results which showed that the p value of the model was less than 0.05 indicating statistical significance (Table 3). The final response function for predicting ethanol production during fermentation is expressed by the design protocol as shown in Equation 2.

$$Y = -2.51 + 8.97X_1 - 0.81X_2 + 0.30X_3 - 1.23X_4 + 0.089X_1X_2 - 0.085X_1X_3 - 10.22X_1X_4 + 0.033X_2X_3 + 5.93X_2X_4 - 0.17X_3X_4 - 4.14X_1^2 - 48.77X_4^2 \quad (2)$$

Where X_1, X_2, X_3 and X_4, represent the levels of yeast extract, NH_4NO_3, glucose and KH_2PO_4 in the fermentation medium respectively. The values of ethanol concentration as predicted by Equation (2) are presented in Table 2 alongside the experimental data for comparison. The results of analysis of variance (ANOVA) carried out to determine the fit of the statistical model are presented in Tables 3 and 4.

Table 2: Central composite design matrix for the optimisation of variables and the response values

Run No	Factors								Response	
	Coded values				Actual values				Ethanol produced (%v/v)	
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	Experiment	Predicted
1	0	0	2	0	0.75	0.28	10	0.06	3.22	3.28
2	0	0	0	0	0.75	0.28	6	0.06	2.34	2.35
3	2	0	0	0	1.00	0.28	6	0.06	2.47	2.52

4	-1	-1	1	-1	0.63	0.16	8	0.03	2.97	2.97
5	-1	1	-1	1	0.63	0.39	4	0.08	1.13	1.09
6	1	-1	1	1	0.88	0.16	8	0.08	2.65	2.61
7	-2	0	0	0	0.50	0.28	6	0.06	1.71	1.66
8	0	0	0	2	0.75	0.28	6	0.10	1.51	1.49
9	1	1	-1	1	0.88	0.39	4	0.08	1.42	1.51
10	0	0	-2	0	0.75	0.28	2	0.06	1.41	1.42
11	0	0	0	0	0.75	0.28	6	0.06	2.25	2.35
12	-1	1	1	-1	0.63	0.39	8	0.03	2.82	2.81
13	1	1	1	-1	0.88	0.39	8	0.03	3.24	3.26
14	0	0	0	-2	0.75	0.28	6	0.01	2.99	3.01
15	1	1	1	1	0.88	0.39	8	0.08	2.39	2.40
16	0	0	0	0	0.75	0.28	6	0.06	2.36	2.35
17	1	-1	1	-1	0.88	0.16	8	0.03	3.46	3.41
18	1	-1	-1	1	0.88	0.16	4	0.08	1.83	1.75
19	-1	1	-1	-1	0.63	0.39	4	0.03	1.68	1.81
20	0	0	0	0	0.75	0.28	6	0.06	2.40	2.35
21	-1	-1	1	1	0.63	0.16	8	0.08	2.37	2.28
22	-1	-1	-1	1	0.63	0.16	4	0.08	1.27	1.34
23	0	0	0	0	0.75	0.28	6	0.06	2.40	2.35
24	0	-2	0	0	0.75	0.05	6	0.06	2.40	2.55
25	0	2	0	0	0.75	0.50	6	0.06	2.34	2.15
26	-1	1	1	1	0.63	0.39	8	0.08	1.94	2.07
27	-1	-1	-1	-1	0.63	0.16	4	0.03	2.09	2.00
28	0	0	0	0	0.75	0.28	6	0.06	2.37	2.35
29	1	1	-1	-1	0.88	0.39	4	0.03	2.34	2.34
30	1	-1	-1	-1	0.88	0.16	4	0.03	2.56	2.52

ANOVA result presented in Table 3 shows a model F – value of 111.96 and a p value less than 0.0001. This implies that the model is significant and also that there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Lack of Fit" p value of of 0.0829 implies it is non-significant. A non-significant lack of fit is good because it is an indication of adequate model fit. The model's coefficient of determination (R^2) was 0.983 while the adjusted R-Squared value was 0.975 as shown in Table 4. The predicted R-Squared value of 0.956 is in reasonable agreement with the adjusted R-Squared value of 0.975. The low value of the coefficient of variation (CV) indicates that the runs were carried out with high precision and reliability (Hou and Chen, 2008). The adequate precision was greater than the recommended minimum value of 4 showing that the model can be used to navigate the design space (Cao et al., 2009).

Table 3: Analysis of variance (ANOVA) for quadratic model

Sources	Sum of Squares	df	Mean Squares	F value	p value
Model	10.11	10	1.01	111.96	< 0.0001
X_1	1.10	1	1.10	121.91	< 0.0001
X_2	0.23	1	0.23	25.70	< 0.0001
X_3	5.17	1	5.17	572.64	< 0.0001
X_4	3.47	1	3.47	383.79	< 0.0001
X_1X_3	0.0072	1	0.0072	0.80	0.3822
X_2X_3	0.0009	1	0.0009	0.10	0.7557
X_2X_4	0.0036	1	0.0036	0.40	0.5353
X_3X_4	0.0009	1	0.0009	0.10	0.7557
X_1^2	0.12	1	0.12	13.18	0.0018
X_4^2	0.017	1	0.017	1.92	0.1819
Residual	0.17	19	0.009		
Lack of Fit	0.16	14	0.011	3.59	0.0829
Pure Error	0.016	5	0.0031		
Cor Total	10.28	29			

Table 4: Statistical information for ANOVA

Parameter	Value
R^2	0.983
Adjusted R^2	0.975
Predicted R^2	0.956
Mean	2.280
Standard deviation	0.095
CV %	4.170
Adequate Precision	40.203

3.2. Effect of Independent Variables on Ethanol Production

The effect of the variables on the response (ethanol concentration) is shown in the response surface and contour plots presented in Figures 1 to 3. The plots which were generated from the statistical model aids in understanding the interactions between the factors and also to locate the optimum levels by varying the values of two variables with the response while keeping the other two variables constant. Figure 1 shows the effect of glucose and NH_4NO_3 on the ethanol concentration. The level of glucose in the fermentation medium had a positive effect on ethanol production. The trend observed indicates that ethanol production was favoured at high glucose levels which is seen from the increase in ethanol concentration when the glucose level was increased. The increase in ethanol concentration in the course of fermentation could be ascribed to the consumption of glucose by the *Saccharomyces cerevisiae* cells to produce ethanol (Ocloo and Ayernor, 2000). A similar trend was reported by Asli (2010) for the production of ethanol from Siahe sardasht grape pomace. Nitrogen sources that are widely used to stimulate ethanol production during fermentations are

ammonium salts, urea, yeast extract and peptones (Laopaiboon et al., 2009). These supplements are typically employed to enhance yeast growth, viability and the rate of sugar utilization, as well as reducing fermentation time. However, several investigators have reported negative effects of using ammonium and urea as nitrogen supplements in ethanol fermentation. A similar observation was obtained in this study as NH_4NO_3 had an overall negative effect on ethanol production. Low levels of NH_4NO_3 was favourable for ethanol production. On the contrary, high levels of NH_4NO_3 will only serve to enhance cell growth and production of biomass and not an increase in ethanol production (Ali and Zulkali, 2011).

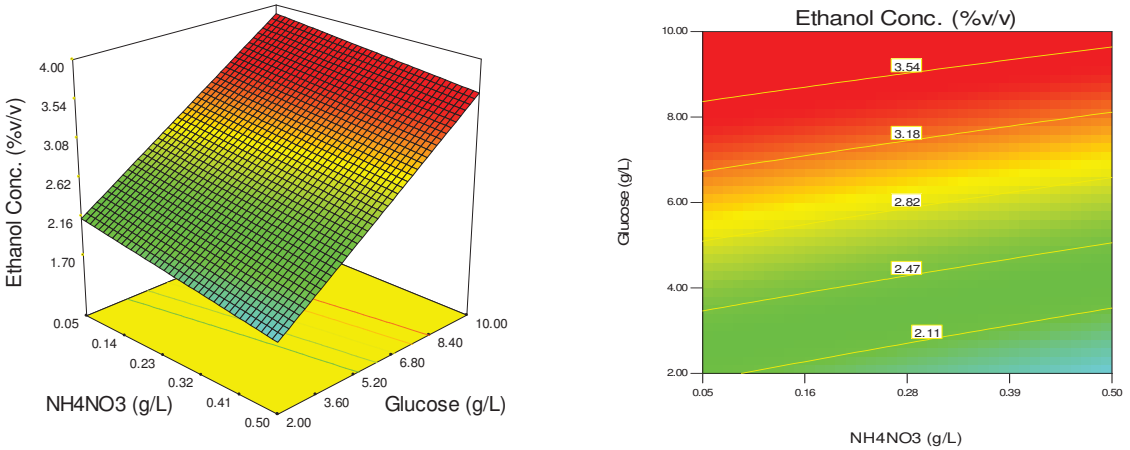

Figure 1: Response surface and contour plot showing the effect of NH_4NO_3 and glucose on ethanol production

Figure 2 shows the effect of KH_2PO_4 on the ethanol concentration. Ethanol produced increased with decrease in KH_2PO_4 showing that relatively low concentrations of phosphorus were favourable for ethanol production. The trend observed with KH_2PO_4 is similar to that observed with NH_4NO_3 on ethanol concentration. Thongdumyu et al. (2014) reported that at relatively low levels of KH_2PO_4, optimum ethanol production could be attained. KH_2PO_4 is however an important medium component. Because of the potassium and phosphate components of KH_2PO_4, it can also be considered to be growth-enhancing as well as serving as a buffering agent by keeping the pH of the medium at the desired value (Ali and Zulkali, 2011).

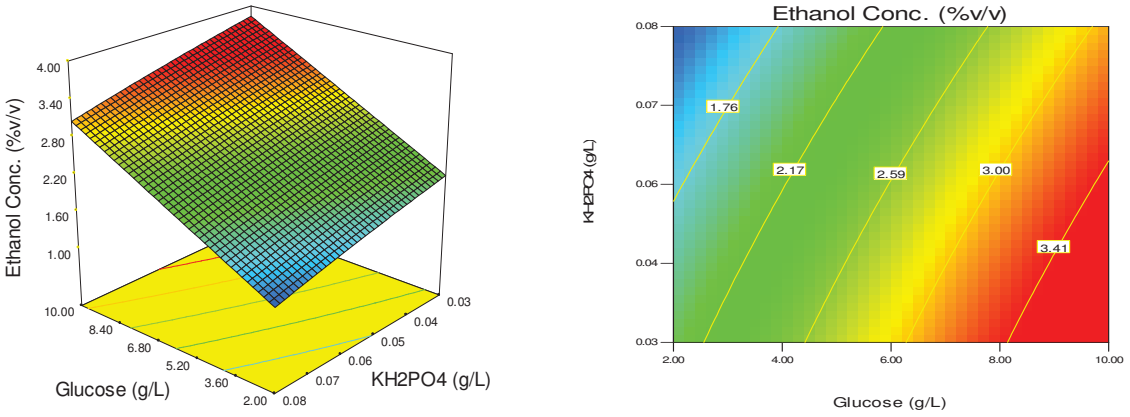

Figure 2: Response surface and contour plot showing the effect of glucose and KH_2PO_4 on ethanol production

Yeast extract have a positive effect on ethanol production as shown in Figure 3. This is seen in the increase in the amount of ethanol produced when the level of yeast extract was increased within the range studied. Similar observations were reported by Chniti et al. (2015) who investigated the effect of nitrogen source on ethanol production from syrup dates by *Saccharomyces cerevisiae*. They reported that the addition of yeast extract to the nutrient medium enhanced ethanol production. Similarly, Turhan et al. (2010) reported enhanced ethanol production from the fermentation of carob extract supplemented with yeast extract. The efficiency of yeast extract in enhancing ethanol production might not be unconnected with its constituents. Yeast extract comprises the water soluble components of the yeast cell, including primarily amino acids, peptides, carbohydrates and salts. Since it is vitamins and other growth stimulating compounds, it therefore has an impact on ethanol production (Chniti et al., 2015).

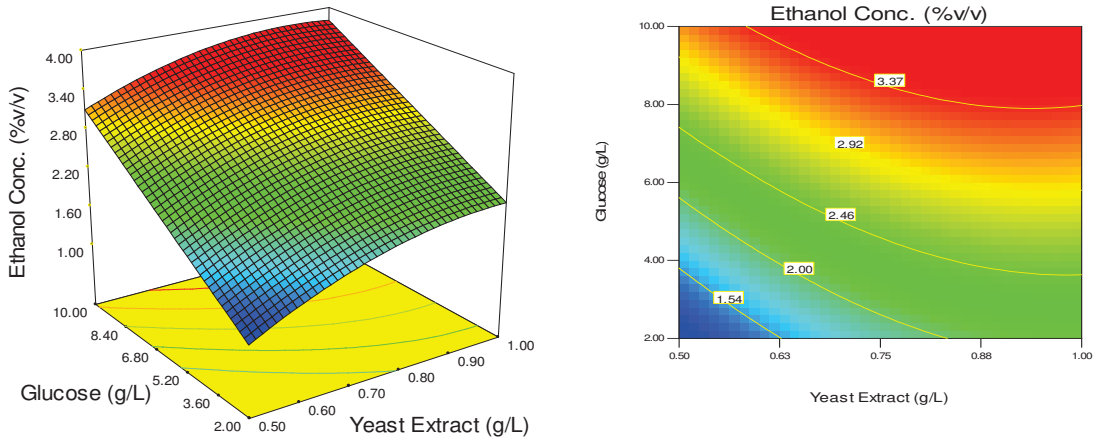

Figure 3: Response surface and contour plot showing the effect of glucose and yeast extract on ethanol production

3.3. Numerical Optimization

Results obtained from numerical optimisation carried out using the Design Expert software revealed that the optimal ethanol concentration was 4.19 %v/v. This was obtained with a nutrient medium having a composition of yeast extract 0.90 g, NH_4NO_3 0.05 g, glucose 10.0 g and KH_2PO_4 0.01 g.

3.4. Validation of Statistical Model

To confirm the validity of the statistical experimental strategy and to gain a better understanding of ethanol production from rice husk hydrolysate, a confirmation experiment with triplicate set was performed at the specified optimum condition representing the maximum point of the concentration of ethanol. Experiments conducted at the optimum condition showed that the ethanol concentration (4.17 %v/v) was closer to the predicted value (4.17 %v/v). The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point.

4. CONCLUSION

This study demonstrated the production of bioethanol from rice husk biomass using saccharomyces cerevisiae. A four-variable central composite design was used to study the simultaneous effect of nutrients: yeast extract, NH_4NO_3, glucose and KH_2PO_4 on the production of ethanol. A statistically significant model ($p<0.0001$) was developed to describe the relationship between ethanol concentration and the chosen independent variables. RSM was used to optimize the process. The optimum values were yeast extract 0.90 g, NH_4NO_3 0.05 g, glucose 10.0 g and KH_2PO_4 0.01 g. At these conditions, maximum ethanol concentration was 4.19 %v/v. Ethanol production was favoured when higher amount of glucose and yeast extract were used while the reverse was the case for the limiting nutrients NH_4NO_3 and KH_2PO_4.

5. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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