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Optimization of Enzymatic Hydrolysis of *Manihot esculenta* Root Starch by α-Amylase and Glucoamylase Using Response Surface Methodology

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Abstract
Cassava starch hydrolysis was investigated in this study using alpha amylase and glucoamylase. The effects of process variables, namely: temperature, pH and time were studied and optimized for hydrolysis of cassava (Manihot esculenta) flour to glucose syrup. Three levels of process variables were used for the study. The three levels of process variables were: temperature (60, 67 and 74 ^{o}C), time (1.5, 2.0 and 2.5 h) and pH (4.5, 5 and 5.5). A polynomial regression model was developed using the experimental data. The results showed that production of reducing sugar was strongly affected by the variation of variables on alpha amylase and glucoamylase hydrolysis of cassava starch. The fit of the model was expressed by the coefficient of determination R^2 which was found to be 0.948 indicating that 94.8 % of the variability in the response can be explained by the model. The value also indicates that only 5.2 % of the total variation is not explained by the model. This shows that equation (2) is a suitable model to describe the response of the experiment pertaining to reducing sugar production. The statistical significance of the model was validated by F-test for analysis of variance ($p \leq 0.05$). For alpha amylase and glucoamylase hydrolysis, the optimum value of temperature, time and pH were found to be 74 ^{o}C, pH 5.5 and time 1.5 h. The maximum reducing sugar production at optimum condition was 257 g/l representing 73.43 % conversion or 73.43 dextrose equivalent (DE).
Key words: cassava starch, hydrolysis, factorial design, glucose syrup, dextrose equivalent.

1. Introduction

Starchy substances constitute the major part of plants, example for plants with high starch content are corn, potato, rice, sorghum, wheat, and cassava. *Manihot esculenta* (Cassava) is a tuberous edible plant of the spurge family. This is the only member of the spurge family that provides food. Saccharification and fermentation of cassava (*Manihot esculenta*) bagasse is the primary step in production of Lactic acid, maltose high fructose corn syrup and bioethanol [1, 2]. Starch is the most abundant form of storage polysaccharides in plants and constitutes an inexpensive source for production of syrups containing glucose, fructose or maltose, which are widely used in food industries [3]. In starch granules, the molecules are densely packed in a polycrystalline state with inter and intramolecular bonds and are hence insoluble in coldwater and often resistant to chemicals and enzymes [4]. In order to make use of the carbon and energy stored in starch, α-amylase enzyme used to break down the polymer to smaller sugar units, which is eventually converted to the individual basic glucose units [5-7]. The bacterial alpha-amylase randomly attacks only the alpha-1,4 bonds. On the other hand, the glucoamylase used in the experiments, attacks the second linkage from the non reducing terminals of the straight segment, release a maltose unit, which is comprised of two glucose units [8]. As most enzymes are water-soluble, they are usually immobilized into insoluble matrices. Various types of immobilization techniques are available, but the cross linking technique in particular involves the addition of similar size of immobilized gels to the starch which is the primary disadvantage in other type of immobilization. Polyacrylamide (PAA) gels have been widely used for the matrix of electrophoresis and they have found applications as support for enzyme immobilization [9, 10]. The enzymatic susceptibility of starch granules has been studied by various authors [l1, 12, 13, 14, 15, 16]). The susceptibility and mode of enzyme action depend on the starch source and enzyme system. The percentage of enzymatic hydrolysis increased when the granules were incubated with alpha-amylase and amyloglucosidase and that the granule susceptibility was affected by granule size and concentration of the hydrolysis products [16]. Starches that naturally show a porous surface, as in the corn, were degraded more easily than those with a smooth surface. According to [1l], the enzymatic susceptibility was not related to the granule size. It had been found for different starches and under various hydrolysis conditions, that

small starch granules are hydrolyzed more than larger ones [17, 18, 19, 20]. The objective of the present work is to investigate hydrolysis of cassava starch with α-Amylase and Glucoamylase with a view to optimize the process variables.

Response Surface Methodology (RSM) is a statistical technique, based on the fundamental principles of statistics, randomization, replication and, duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments. These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and noncontrollable influences [21, 22].

2. Material And Methods

2.1 Preparation of cassava flour

Fresh cassava tubers obtained from a daily market in Ede, Osun State, Nigeria were peeled and washed with tap water, chipped into small sizes and dried at 60 ^{o}C for 48 h in a cabinet dryer. The dried chips were dry-milled and screened to produce the flour.

2.2 Cassava flour hydrolysis with α-Amylase and Glucoamylase

A 17.5 kg (35 % slurry) Cassava flour was dispatched into the hydrolyzer and cooked at 98 ^{o}C for about 10min so as to gelatinize the starch and a quantity of Termamyl the heat stable α-amylase from *Bacilus licheniformis* (equivalent to 0.5ml/kg starch) was added and allowed to remain at this condition for five minutes to thin out. It was cooled down to 72 ^{o}C for another 25 min. The mixture was further cooled down to 60 ^{o}C when a quantity of amyloglucosidase (equivalent to 0.5 ml/kg starch) was also added and allowed to remain at this condition for 2 h. The temperature, pH and time were varied on the basis of statistical experimental design. The range and the levels of process variables under study are given in Table 1. Samples were withdrawn regularly and filtered after stopping the enzymatic activities by boiling for 15 min and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller [23]. These procedures were carried out in triplicate and only the average values are reported.

2.3 Statistical Analysis for Experimental Design

In order to maximize the glucose production, full factorial design for three independent variables was adopted. Full factorial design was used to obtain the combination of values that can optimize the response within the region of the three dimensional observation spaces, which allows one to design a minimal number of experimental runs. The variables were temperature, time and pH for this study. The actual values of the variables at coded levels -1, 0 and + 1 are given in Table 1. The selection of low, middle and high levels for all these variables were based on a prior screening done in the laboratory (unpublished data). A 2^3 full factorial design with 2 replicates at the center point, leading to the total number of 10 experiments. The behaviour of the present system described by the following equation (1), which includes all interaction terms regardless of their significance

Table 1: Optimization of physical condition for the production of reducing sugar by α-amylase and glucoamylase.

Variables	Parameter	Coded level		
		-1	0	1
x_1	Temperature(^{o}C)	60.0	72.0	74.0
x_2	pH	4.5	5.0	5.5
x_3	Time (h)	1.5	2.0	2.5

Independent variables in a 2^3 full factorial experiment design. The parameters used in this experiment are temperature, pH and time.

$$Y_n = b_o + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

Where Y_n is predicted response, i.e yield of glucose; x_1, x_2 and x_3 are independent variables; b_o is coefficient constant for offset term; b_1, b_2 and b_3 are coefficient constant for linear effects and b_{12}, b_{13}, b_{23} are coefficient constant for interactions effects. The variables studied using α-amylase and glucoamylase medium were temperature (60-74 ^oC), pH (4.5-5.5) and time (1.5-2.5 h). Regression model containing three linear, three interaction terms and one block term was employed by using statistical software (SAS 9.1). The model evaluates the effect of each independent variable to a response.

3. Result and Discussions

The production of reducing sugar at each experimental run is given in Table 2 along with model predicted values. The results were analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The regression equations obtained, after analysis of variance, gave the level of production of reducing sugar as a function of different variables: temperature, pH and time. All terms regardless of their significance are included in the following equation:

$$Y_n = 299.9399 - 0.9558x_1 - 12.8686x_2 + 4.3909x_3 + 0.2438x_1x_2 - 0.0369x_1x_3 - 0.4233x_2x_3 \quad (2)$$

where Y_n is the response, that is, reducing sugar production (g/l) and x_1, x_2 and x_3 are the test variables: temperature, pH and time, respectively. According to equation (2) all the factors have positive effects, except temperature (x_1), pH (x_2) and interaction between temperature-time (x_1x_3). The fit of the model was expressed by the coefficient of determination R^2 which was found to be 0.974 indicating that 97.4 % of the variability in the response can be explained by the model. The value also indicated that only 2.6 % of the total variation was not explained by the model. This showed that equation (2) is a suitable model to describe the response of the experiment pertaining to reducing sugar production. The value of the adjusted determination coefficient (Adj =0.968) is also very high to advocate for a high significance of the model. A high value of the correlation coefficient (R= 0.987), justifies an excellent correlation between the independent variables. This indicates good agreement between the experimental and predicted values of reducing sugar production as shown in Table 2. The best reducing sugar production was observed when temperature of 74 ^oC, pH of 5.5 and time of 1.5 h were used in the production. Results were the average of triplicate analysis.

The corresponding analysis of variance (ANOVA) is presented in Table 3. Statistical testing of the model can also be done by the Fisher's statistical test for analysis of variance. The F value is the ratio of the mean square due to regression to the mean square due to the

Table 2 Experimental and model predicted values of reducing sugar concentration in α-amylase and glucoamylase hydrolysis.

Run	Temperature (^oC), x_1	pH x_2	Time (h) x_3	Average Response (g/l)	Standard Deviation	Predicted value (g/l)
1	60	4.5	1.5	251.04	0.236925	250.92
2	60	4.5	2.5	251.18	0.25632	251.19
3	60	5.5	1.5	252.03	0.215019	252.04
4	60	5.5	2.5	252.01	0.264575	251.89
5	67	5.0	2.0	253.09	0.285365	253.78
6	67	5.0	2.0	253.07	0.306649	253.78
7	74	4.5	1.5	252.03	0.487476	252.12
8	74	4.5	2.5	252.25	0.277128	251.88
9	74	5.5	1.5	257.03	0.232881	256.66
10	74	5.5	2.5	256.24	0.070238	255.99

real error. Generally, the calculated F value should be several times the tabulated value if the model is a good predictor of the experimental results. The response taken from Table 3 revealed that the linear term of temperature (x_1), pH (x_2) and time (x_3) have remarkable effects on the reducing sugar yield. The significance of each coefficient was determined using p-value ($p < 0.05$) and the smallest p-value indicates high significance of the corresponding coefficient. It can be seen that the variables with the largest effect was temperature. All the linear terms and interaction terms are significant.

The response surface plots and process variables are shown in Figures 1-3. These response surface plots help in assessing the combined effect of any two variables on the yield of reducing sugar. Thus the effects of pH-temperature, time-temperature and time-pH on the response can be obtained. The orange colour means higher reducing sugar yield in Figures 1-3. Fig.1 shows the interaction effect of pH and temperature on reducing sugar yield while the time is kept constant at 1.5 h. It was observed that at high level of pH (5.2-5.5) and at high level of temperature (73-74 ^oC) the reducing sugar yield was high (>257 g/l). The significance

Table 3. Regression analysis (ANOVA) for the production of reducing sugar by α-amylase and glucoamylase.

Factor	SS	DF	MS	F	P-value
Temp. (x_1)	15.9330	1	15.9330	352.06	0.0028
pH (x_2)	14.6070	1	14.6070	322.76	0.0031
Time (x_3)	0.0253	1	0.0253	0.56	0.5325
x_1 by x_2	6.4261	1	6.4261	141.99	0.0070
x_1 by x_3	0.0595	1	0.0595	1.32	0.3702
x_2 by x_3	0.1711	1	0.1711	3.78	0.1913
Error	0.0905	3	0.0453		
Total	37.3097	9			

R- coefficient of correlation =0.974; R^2 - coefficient of determination = 0.948; Adjusted R^2 = 0.934. SS: sum of squares; DF: Degree of freedom; MS: square means.

of linear effects of pH (p=0.003) and temperature (p=.0.003) and interaction effect of this {x_1x_2 (p=0.007)} were evident from Table 3. Fig.2 shows the interaction effect of time and temperature on reducing sugar yield while the pH is kept constant at 5.5. It was observed that at low level of time (1.5-1.8 h) and at middle to high level of temperature (72-74 ^oC) the reducing sugar yield was high (>255 g/l).

The significance of linear effect of temperature (p=0.003) and the negative effect of this interaction (negative coefficient for x_1x_3) were evident from Table 3 and modelling equation (2) respectively. Fig.3 shows the interaction effect of time and pH on reducing sugar yield while the temperature is kept constant at 74 ^oC. It was observed that at low to middle level of time (1.5-2.2 h) and at high level of pH also (5.2-5.5) the reducing sugar yield was high (>255 g/l). The significance of linear effects of pH (p=0.003) and the negative effect of this interaction (negative coefficient for x_2x_3) were also evident from Table 3 and modelling equation (2) respectively. The optimal values of pH, temperature and time estimated in actual units were 5.5, 74 ^oC, 1.5 h, respectively with predicted reducing sugar yield of 256.66 g/l. The experimental value for these predicted optimum conditions for reducing sugar yield was obtained to be 257.03 g/l, which was 0.1% higher than the predicted value, which reveals a high accuracy of the model.

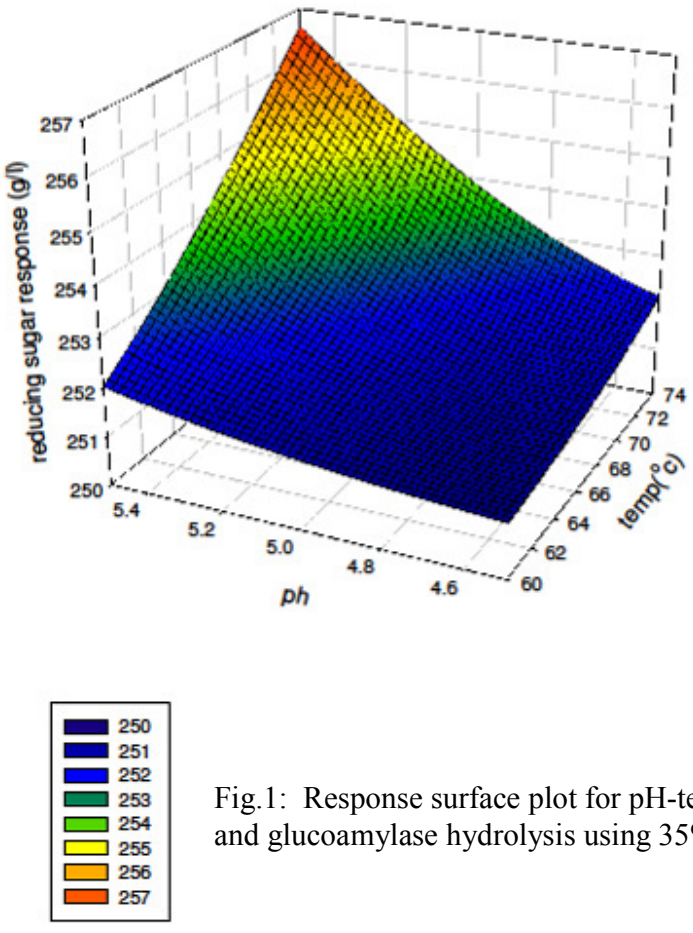

Fig.1: Response surface plot for pH-temperature in α-amylase and glucoamylase hydrolysis using 35% cassava flour slurry.

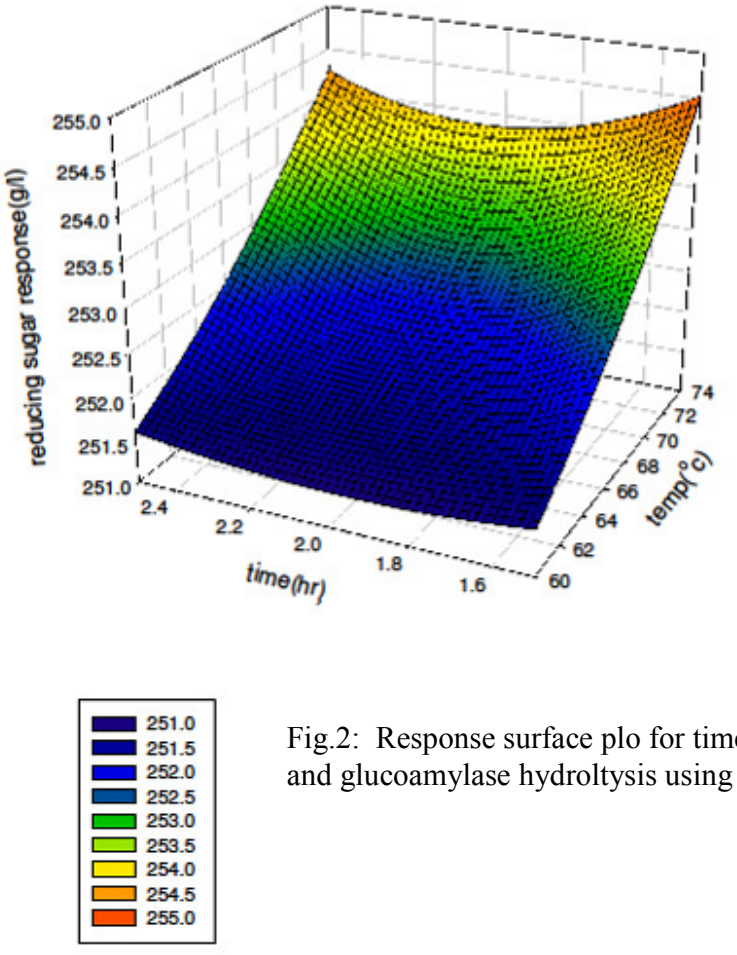

Fig.2: Response surface plo for time-temperature in α-amylase and glucoamylase hydroltysis using 35% cassava flour slurry.

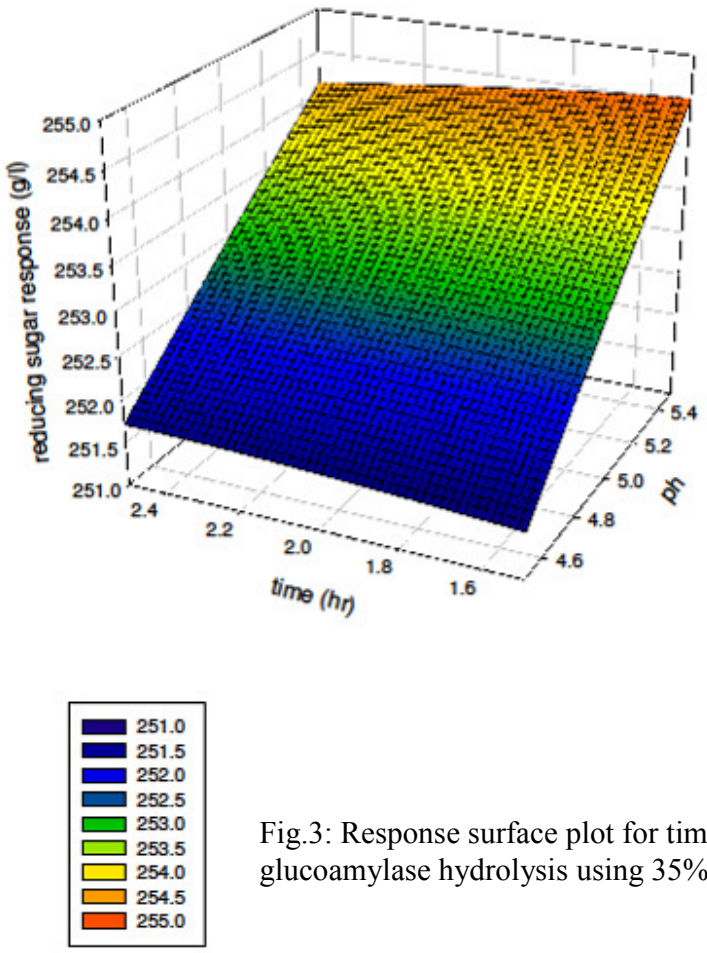

Fig.3: Response surface plot for time-pH in α-amylase and glucoamylase hydrolysis using 35% cassava flour slurry.

4. Conclusions

The analysis using full factorial design revealed that maximum reducing sugar production in α-amylase and glucoamylase hydrolysis can be achieved only at time of 1.5 h. The study revealed that temperature has the most significant effect on the reducing sugar production. Therefore, at time of 1.5 h, pH of 5.5 and 74 ^{o}C, the highest reducing sugar was achieved and the maximum reducing sugar production at the optimum condition was 257 g/l representing 73.43 % conversion.

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