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Optimization of Enzymatic Hydrolysis of *Manihot Esculenta* Root Starch by Immobilized α-Amylase Using Response Surface Methodology

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***Abstract*—**Enzymatic hydrolysis of starch from natural sources finds potential application in commercial production of alcoholic beverage and bioethanol. In this study the effect of starch concentration, temperature, time and enzyme concentration were studied and optimized for hydrolysis of cassava *(Manihot esculenta)* starch powder (of mesh 80/120) into glucose syrup by immobilized (using Polyacrylamide gel) α-amylase using central composite design. The experimental result on enzymatic hydrolysis of cassava starch was subjected to multiple linear regression analysis using MINITAB 14 *softwar*e. Positive linear effect of starch concentration, enzyme concentration and time was observed on hydrolysis of cassava starch by α-amylase. The statistical significance of the model was validated by F-test for analysis of variance ($p \leq 0.01$). The optimum value of starch concentration temperature, time and enzyme concentration were found to be 4.5% (w/v), 45^{o}C, 150 min, and 1% (w/v) enzyme. The maximum glucose yield at optimum condition was 5.17 mg/mL.

***Keywords*—**Enzymatic hydrolysis, Alcoholic beverage, Central composite design, Polynomial model, glucose yield.

I. 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 L-lactic acid, maltose high fructose corn syrup and bioethanol [1,2]. 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 [3-5]. The bacterial alpha-amylase randomly attacks only the alpha-1,4 bonds. On the other hand, the fungal alpha-amylase 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 [6].

As most enzymes are water-soluble, they are usually immobilized into insoluble matrices. There are 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 [7,8].

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 non-controllable influences [9,10].

In this study central composite design was applied for optimization of process parameters like starch concentration, enzyme concentration, temperature, and time which affects the kinetics of cross linked α -amylase catalyzed hydrolysis of cassava starch.

II. EXPERIMENTAL

A. Cassava (Manihot Esculenta) root starch

The Cassava *(Manihot Esculenta)* root used in this present work was purchased from local market (Tamilnadu, India) as raw cassava. The outer layer of the cassava roots was removed and the inner part was chipped into small pieces, dried in oven at 65ºC for 24 h to remove moisture and powdered. The starch powder screened using 80/120 was used in all experiments. The cassava starch solution used through out the study was

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prepared by dissolving required quantity (based on table I) in distilled water and gently boiled and pH was adjusted to 3 (optimized by preliminary study).

B. *Aspergillus oryzae α-amylase*

The fungal 1,4-Alpha-D-glucan- glucanohydrolase (α-amylase CAS NO. 9001-19-8, 1:2000 IP Units) produced from *Aspergillus oryzae* source used in the present study was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India.

C. *Immobilization of α-amylase by gel-entrapment*

PAA microgel particles of fixed size have been prepared using the following composition. 10 ml of buffered monomer solution contains 116.8 mg monomeric acrylamide and 3.6 mg of N,N -bisacrylamide solution, added with 1.5 mL tris-HCl prevent enzyme denaturation, 120 µL of 10% SDS and 40 µl of N,N,N,N-tetramethylethylenediamine (TEMED) as the activator, The required amount of enzyme (based on table 2) was added and the mixture was homogenized under magnetic stirring and purged with nitrogen to remove residual oxygen mixed well. Finally add 120 µL of 10 % ammonim per-sulphate to initiate polymerization. After 60 min of polymerization at room temperature the microgel was cut into similar size particles (2×2×2 mm). Microgel particles were washed with Tris-HCl buffer to remove residual solvent and water then used for hydrolysis of cassava starch [7,8].

D. *Optimization by Response Surface Methodology*

Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations existing between cluster of controlled experimental factors and the measured responses. In this work, effect of four variables to be evaluated, the recommended number of tests at the centre point was six and therefore the total number of experiment was 31. The Values of the variables are coded to lie ±1 for factorial points, 0 for the center points and ± 2 for axial points.

Four variables, which were expected to have an effect on hydrolysis of starch using the immobilized α-amylase were identified by a preliminary research. The range and the actual levels of the variables investigated in this study are given in table I. The variables considered for the design were: Substrate concentration % (w/v) (X_1), Enzyme concentration % (w/v) (X_2), Temperature °C (X_3) & Time in min (X_4) and central composite experimental design (Table II) was developed using Minitab14 *software.* The effect of these 4 variables on enzymatic hydrolysis of cassava starch fit to the second order polynomial model to these four independent variables according to equation 1.

$$Y=b_0 + \Sigma b_i X_i + \Sigma b_{ii} X_i^2 + \Sigma\Sigma b_{ij} X_i X_j \quad (1)$$

Where Y is the response variable to be modeled, x_i and x_j are the independent variables and b_i, b_{ii}, b_{ij} are the measures of the x_i, x_j and x_i x_j effects respectively. The variables xixj represents the first-order interactions between x_i and x_j [9-11]. The optimum values of the selected variables were obtained from the estimated variables in the model and by inspecting the response surface contour plots and MINITAB 14 optimizer.

TABLE I
PROCESS VARIABLES IN CODED AND ACTUAL UNITS

Variable	coded level				
	-2	-1	0	+1	+2
Substrate concentration (X_1), % (w/v)	1.5	3	4.5	6	7.5
Enzyme concentration (X_2), % (w/v)	0.5	0.75	1	1.25	1.5
Temperature (X_3), °C	35	45	55	65	75
Time (X_4), min	60	90	120	150	180

TABLE II
CENTRAL COMPOSITE EXPERIMENTAL DESIGN FOR FOUR VARIABLES AND EXPERIMENTAL AND PREDICTED GLUCOSE YIELD

Std Order	X_1.	X_2	X_3	X_4	Glucose yield (mg/mL)	
					Experimental	Predicted
1	-1	-1	-1	-1	2.608	2.216
2	1	-1	-1	-1	4.175	3.650
3	-1	1	-1	-1	4.732	4.527
4	1	1	-1	-1	5.222	5.129
5	-1	-1	1	-1	0.541	0.960
6	1	-1	1	-1	1.812	1.650
7	-1	1	1	-1	2.133	3.057
8	1	1	1	-1	2.130	2.915
9	-1	-1	-1	1	4.464	3.637
10	1	-1	-1	1	5.630	4.654
11	-1	1	-1	1	4.726	4.835
12	1	1	-1	1	5.484	5.021
13	-1	-1	1	1	1.535	1.576
14	1	-1	1	1	1.687	1.850
15	-1	1	1	1	2.079	2.561
16	1	1	1	1	1.662	2.002
17	-2	0	0	0	3.554	3.232
18	2	0	0	0	3.687	4.107
19	0	-2	0	0	0.732	1.815
20	0	2	0	0	5.264	4.279
21	0	0	-2	0	2.318	3.958
22	0	0	2	0	1.226	0.316
23	0	0	0	-2	4.191	3.769
24	0	0	0	2	3.757	4.277
25	0	0	0	0	4.468	4.644
26	0	0	0	0	4.942	4.644
27	0	0	0	0	4.468	4.644
28	0	0	0	0	4.847	4.644
29	0	0	0	0	4.468	4.644
30	0	0	0	0	4.847	4.644
31	0	0	0	0	4.468	4.644

For statistical calculations, the variables were coded according to equation 2.

$$xi = X_i - X_0 / (\Delta X_i) \quad (2)$$

Where, xi is the independent variable coded value, X_i is independent variable real value, X_0 is independent variable real value on the centre point and ΔX_i is the step change value.

E. Estimation of Glucose

Experiments were conducted according to central composite design (Table II) to study the effect of cassava starch concentration, temperature, enzyme concentration and time on enzymatic hydrolysis of starch by immobilized α-amylase enzyme. All the experiments were conducted in 100 mL mixed reactor constantly mixed by magnetic stirrer. The glucose yield (Y) from hydrolysis of cassava starch was estimated using glucose oxidase-peroxidase kit (GOD/POD) based on trinder's method [12].

III. Results and Discussion

Experimental values of Glucose yield (mg/mL) given in table II were subjected to multiple linear regression analysis using MINITAB 14 software. In order to approach the optimal response region of the glucose yield (mg/ml) from hydrolysis of cassava starch by α-amylase enzyme, the effect of temperature, cassava starch concentration, enzyme concentration and time on hydrolysis described in the form equation 3, a second order polynomial model in coded units.

$$Y_{\text{glucose yield}} = 4.64422+0.21882X_1+0.61584X_2-1.06860X_3+0.12699X_4-0.24369X_1^2-0.39935X_2^2+0.70587X_3^2-0.15532X_4^2-0.20800X_1X_2-0.18611X_1X_3-0.10410X_1X_4-0.05354X_2X_3-0.27807X_2X_4-0.20123X_3X_4 \quad (3)$$

The student's t-test was performed to determine the significance of the regression coefficients. The results of statistical analysis including the regression coefficient, t and p values for linear, quadratic and combined effects of the variables were given in the table III. The larger the magnitude of the t-value and the smaller the p-value, indicate more significant of the corresponding coefficient and its effect on hydrolysis of cassava starch by α-amylase. The p-values are used as a tool to check the significance of each of the coefficients and to understand the interactions between the best variables. Positive coefficients for X_1 (Substrate concentration), X_2 (Enzyme concentration) and X_4 (time) indicates a linear effect to increase on hydrolysis of cassava starch by α-amylase, while negative coefficient of X_3 (temperature) revealed the opposite effect. The quadric effect of enzyme concentration and time also had a significant effect ($p<0.05$), but no interactions between the variables found to contribute to the response the glucose yield. Joglekar and May (1987) have suggested for a good fit of a model, regression coefficient R^2 should be at least 80%. The R^2 value is the proportion of variation in the response attributed to the model was 0.824. This means that this model fitted well with the experimental data. The R^2 value implies that the sample variation of 82.4% for glucose yield is attributed to the factors.

The statistical significance of the model was also determined by F-test for analysis of variance (ANOVA) and residuals analysis was performed to validate the model at 99% confidence level. The model fitted well with the Glucose yield and the optimal values from the model was justified ($p=0.001$). The ANOVA given in table IV indicates that the Linear and quadratic terms in second order polynomial Model (equation 3) were highly significant ($p<0.01$) and adequate to represent the relationship between glucose yield (mg/ml) and enzyme concentration, substrate concentration, temperature and time. The response surface plots describing interaction effect between pair of factors on hydrolysis of cassava starch were given in figure 1 to 6 by keeping other two factors constant at their middle level. Fig. 1 shows the interaction effect of starch concentration and enzyme concentration on glucose yield while the temperature and time were kept constant at 55ºC & 120 min respectively. It was observed that at middle level of substrate concentration (5 to 6% (w/v)) and at middle level of enzyme concentration (1% (w/v)) the glucose yield was high (>4.5 mg/mL).

Fig. 2 shows the interaction effect of starch concentration and temperature on glucose yield while the enzyme concentration and time were kept constant at 1% (w/v) & 120 min respectively. It was observed that at middle to higher level of substrate concentration (4 to 8% (w/v)) and at lower to middle level of temperature (40 to 60ºC) the glucose yield was high (>4.5 mg/mL). The negative effect of (negative coefficient for X_3) of temperature at high level was also evident from table III. Fig. 3 shows the interaction effect of temperature and time on glucose yield while the starch concentration and enzyme concentration were kept constant at 4.5% (w/v) and 1% (w/v) respectively. It was observed that at middle to higher level of time (80 to 150 min) and at lower to middle level of temperature (40 to 60ºC) the glucose yield was high (>4.5 mg/mL). The negative interaction effect (negative coefficient for X_3X_4) was also evident from table III. Fig. 4 shows the interaction effect of enzyme concentration and time on glucose yield while the starch concentration and temperature were kept constant at 4.5% (w/v) and 60ºC respectively.

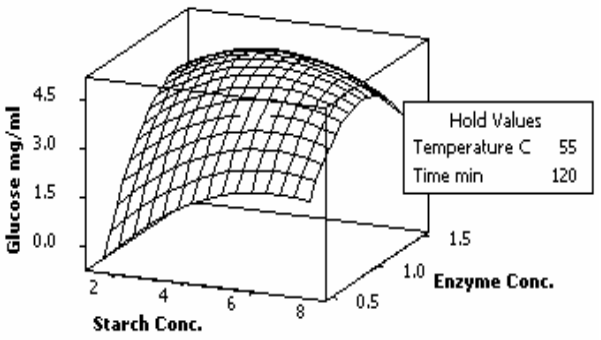

Fig. 1 Response surface plot of the combined effect of starch and enzyme concentration on glucose yield

It was observed that at lower to middle level of temperature (40 to 60ºC) and at middle to higher level of enzyme concentration (1 to 1.5% (w/v)) the glucose yield was high (>5 mg/mL). The significance of positive linear effect of enzyme concentration ($p=0.004$) and interaction effect (negative coefficient for X_2X_4) were also evident from table III.

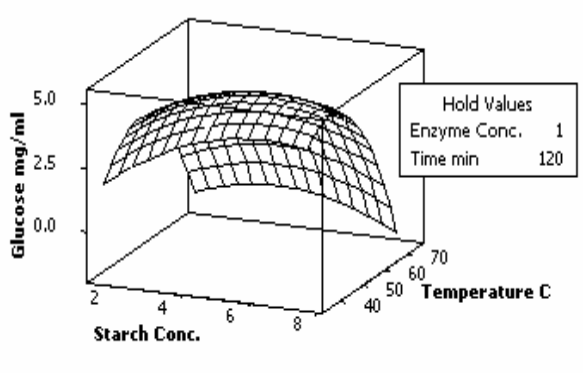

Fig. 2 Response surface plot of the combined effect of starch concentration and temperature on glucose yield

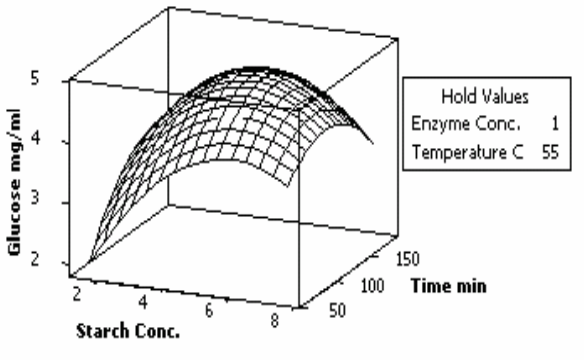

Fig. 3 Response surface plot of the combined effect of starch concentration and time on glucose yield

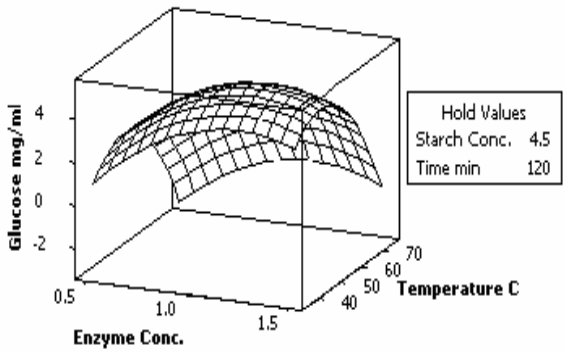

Fig. 4 Response surface plot of the combined effect of enzyme concentration and temperature on glucose yield

Fig. 5 shows the interaction effect of enzyme concentration and temperature on glucose yield while the starch concentration and time were kept constant at 4.5% (w/v) and 120 min respectively. It was observed that at middle to higher level of time (80 to 150 min) and at lower to middle level of temperature (40 to 60ºC) the glucose yield was high (>4 mg/mL). The significance of linear effect of enzyme (p=0.004) and the negative effect of this interaction (negative coefficient for X_2X_3) was also evident from table III. Fig. 6 shows the interaction effect of starch concentration and time on glucose yield while the enzyme concentration and temperature were kept constant at 1% (w/v) and 55ºC respectively.

TABLE III
ESTIMATED REGRESSION COEFFICIENTS OF SECOND ORDER POLYNOMIAL MODEL FOR OPTIMIZATION OF GLUCOSE YIELD

Coefficient	Estimated Coefficient	Standard Deviation	t- value	p-value
β_0	4.644	0.335	13.833	0.000
β_1	0.218	0.181	1.207	0.245
β_2	0.615	0.181	3.396	0.004
β_3	-1.068	0.181	-5.893	0.000
β_4	0.126	0.181	0.700	0.494
β_{11}	-0.243	0.166	-1.467	0.162
β_{22}	-0.399	0.166	-2.404	0.029
β_{33}	-0.705	0.166	-4.249	0.001
β_{44}	-0.155	0.166	-0.935	0.364
β_{12}	-0.208	0.222	-0.937	0.363
β_{13}	-0.186	0.222	-0.838	0.414
β_{14}	-0.104	0.222	-0.469	0.646
β_{23}	-0.053	0.222	-0.241	0.813
β_{24}	-0.278	0.222	-1.252	0.229
β_{34}	-0.201	0.222	-0.906	0.378

TABLE IV
ANALYSIS OF VARIANCE (ANOVA) OF SECOND ORDER POLYNOMIAL MODEL FOR OPTIMIZATION OF GLUCOSE YIELD BY THE HYDROLYSIS OF STARCH

Factor	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Regression	14	59.2757	4.233	5.37	0.001
Linear	4	38.0445	9.511	12.05	0.000
Square	4	17.8806	4.470	5.67	0.005
Interaction	6	3.3507	0.558	0.71	0.648
Residual error	16	12.6247	0.789		
Lack-of-fit	10	12.3293	1.232	25.04	0.000
Pure error	6	0.2954	0.049		
Total sum of squares	30	71.9004			

The optimal values of starch concentration, enzyme concentration, temperature and time was estimated in actual units were 4.5% (w/v), 1 % (w/v), 45°C, and 150 min respectively with predicted Glucose yield (mg/mL) of 5.17 mg/mL (Fig. 7). Confirmation experiment was conducted for these predicted optimum conditions, glucose yield (mg/ml) from experiment obtained was 5.35 mg of glucose/mL, which

was 3.48% higher than the predicted value, which reveals a high accuracy of the model.

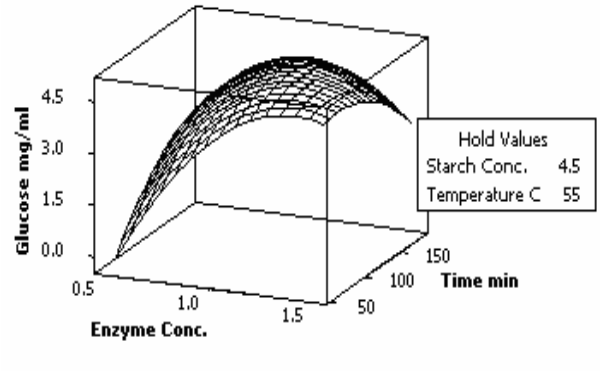

Fig. 5 Response surface plot of the combined effect of enzyme concentration and time on glucose yield

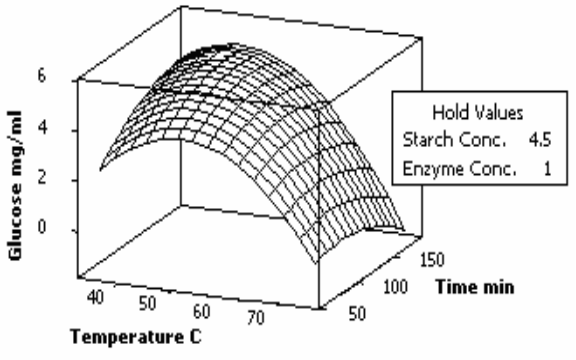

Fig. 6 Response surface plot of the combined effect of temperature and time on glucose yield

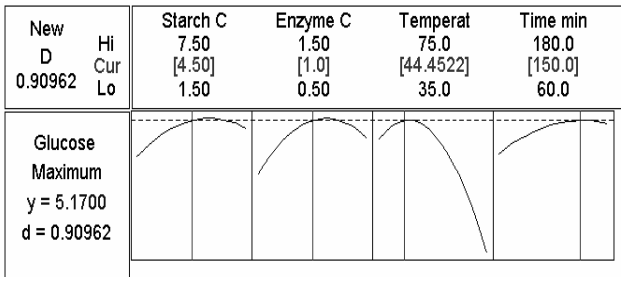

Fig. 7 Optimization plot

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