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Statistical Optimization of Enzymatic Hydrolysis of Potato (*Solanum tuberosum*) Starch by Immobilized α-amylase

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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 Potato starch powder (of mesh 80/120) into glucose syrup by immobilized (using Sodium arginate) α-amylase using central composite design. The experimental result on enzymatic hydrolysis of Potato 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 Potato 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, enzyme concentration, temperature, time and were found to be 6% (w/v), 2% (w/v), 40°C and 80min respectively. The maximum glucose yield at optimum condition was 2.34 mg/mL.

Keywords**—**Alcoholic beverage, Central Composite Design, Enzymatic hydrolysis, Glucose yield, Potato Starch.

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. The conversion of starch to value-added products by enzyme-catalyzed reactions represents the largest industrial use of enzyme molecules. The production of ethanol from starch consists of two steps: hydrolysis of starch molecules and fermentation of the hydrolysis products. Hydrolysis of starch can be achieved using either acid or enzyme catalysts [1].

Amylase has a great deal of application in starch saccharification. The amylolytic enzymes and a wide spectrum of applications in food industry for production of glucose syrups, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilization and saccharification of starch for alcohol fermentation in brewing industries, and also find a wide range of application in baking, paper, textile and detergent industry [2].Many cereal seeds and tubers/roots store starch. Potato is an abundant material that mainly contains (fresh matter w/w): moisture (80 ±2%), starch (18 ±2%), cellulose and hemicelluloses (1.5± 0.5%), glucose (0.4± 0.3%) and proteins (2±1.5%) [3].In most cases the enzymatic process is inhibited by high substrate and product concentration and also instability of the enzyme under repetitive or prolonged use. Immobilization is an important technique for continuous and repeated use of enzymes in industrial application and also rapid separation of the enzyme from the reaction medium. The above features would be important in the development of an economically feasible bioreactor for the starch hydrolysis industry thus immobilizing a-amylase would be of great importance.

The general methods employed for immobilization are entrapment, microencapsulation, and copolymerization, cross linking, physical adsorption, chemical attachment and covalent binding [4-8]. The aim of this work includes the statistical optimization of enzymatic hydrolysis of potato starch that has been predicted to play a very significant role in enhancing the conversion efficiency of starch to glucose. Hence, experimental factorial design and the response surface methodology have already been successfully applied in other fields [9-12]. 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 entrapment α -amylase catalyzed hydrolysis of potato starch.

II. Materials and Methods

A. Potato (Solanum tuberosum) root starch

The Potato *(Solanum tuberosum)* root used in this present work was purchased from local market (Tamilnadu, India) as raw Potato. The outer layer of the potato 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 Potato starch solution used through out the study was prepared by dissolving required quantity (based on Table 2) in distilled water and gently boiled.

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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 Entrapment

The sodium alginate entrapment of enzyme was performed according method of Johnsen and Flink [13]. Sodium alginate solution 3%(w/v) (Loba Chemie, Mumbai, India) was prepared by dissolving sodium alginate in 100ml boiling water and enzyme (based on Table 2) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2 M $CaCl_2$ solution from 5-cm height and kept for curing at 4°C for 1 h. The cured beads were washed with sterile distilled water 3 to 4 times then used for hydrolysis of Potato starch.

D. Optimization by Response Surface Methodology

Independent Variable (Starch concentration, Enzyme concentration and Temperature, Time) were optimization by RSM using CCD of experiments. Table 1 each variable was studied at different levels Starch concentration (X_1, (w/v) %), Enzyme concentration (X_2, (w/v) %), Temperature(X_3, °C) and Time (X_4, min). Experimental Design includes 31 flasks separately for each with replicates. Upon Complete of experiments, Glucose concentration was taken as a dependant variable (or) Response. A full polynomial model obtained by a multiple regression technique for four factors using MINITAB 14 to estimate the response of the dependent variable.

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4 \quad (1)$$

where Y is predicted response, X_1, X_2, X_3, X_4 the independent variables, β_0 the offset term, β_1, β_2, β_3, β_4 the linear effects, β_{11}, β_{22}, β_{33}, β_{44} the squared effects and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are interaction terms.

TABLE I
EXPERIMENTAL RANGE AND LEVELS OF THE INDEPENDENT VARIABLES

Variables	-1	0	+1
Starch conc (w/v)%, X_1	3	6	9
Enzyme conc (w/v)%, X_2	0.5	1	1.5
Temperature (°C), X_3	30	40	50
Time (min), X_4	40	80	120

D. Estimation of Glucose

Experiments were conducted according to central composite design Table1 to study the effect of potato starch concentration, enzyme concentration, temperature, and time on enzymatic hydrolysis of starch by immobilized α-amylase enzyme. All the experiments were conducted in 50 mL mixed reactor constantly mixed by incubation shaker. The glucose yield (Y) from hydrolysis of potato starch was estimated using DNS method [14].

III. RESULTS AND DISCUSSION

Data of the experimental yields for glucose were fitted on to a second-order polynomial equation (1) to generate regression coefficient values Table 3 which exhibited a R^2 value of 0.93. The final predictive equation is given as

$$\text{Glucose yield (mg/ml)} = -4.774+0.444X_1+1.043X_2+0.158X_3+0.001X_4-0.029X_1^2-0.023X_2^2-0.001X_3^2-0.000X_4^2+0.025X_1X_2+0.000X_1X_3-0.000X_1X_4-0.021X_2X_3+0.002X_2X_4+0.000X_3X_4 \quad (2)$$

TABLE II
ESTIMATED REGRESSION COEFFICIENTS OF SECOND ORDER POLYNOMIAL MODEL FOR OPTIMIZATION OF GLUCOSE CONCENTRATION

Coefficient	Estimated Coefficient	t- value	p-value
β_0	-4.774	-4.498	0.000
β_1	0.444	4.478	0.000
β_2	1.043	1.754	0.099
β_3	0.158	4.427	0.000
β_4	0.001	0.228	0.823
β_{11}	-0.029	-6.957	0.000
β_{22}	-0.023	-0.153	0.880
β_{33}	-0.001	-3.376	0.004
β_{44}	-0.000	-0.056	0.956
β_{12}	0.025	0.731	0.476
β_{13}	0.000	0.049	0.962
β_{14}	-0.000	-1.364	0.192
β_{23}	-0.021	-2.070	0.055
β_{24}	0.002	1.145	0.269
β_{34}	0.000	0.365	0.720

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

Factors	Degrees of Freedom	Sum of Squares	Mean Square	F-value	P-value
Regression	14	9.16264	0.65447	15.52	<0.001
Linear	4	6.42030	0.35345	8.38	0.001
Square	4	2.39986	0.59996	14.23	<0.001
Interaction	6	0.34247	0.05707	1.35	0.291
Residual Error	16	0.67454	0.04215		
Lack-of-Fit	10	0.64245	0.06424	12.01	0.003
Pure Error	6	0.03209	0.00534		
Total	30	9.8371			

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 4. 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 potato 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_2 (Enzyme concentration), X_3 (temperature) and X_4 (time) indicates a linear effect to increase on hydrolysis of potato starch by α-amylase, while negative coefficient of X_1 (temperature) revealed the opposite effect. The quadric effect of starch concentration and temperature also square term of starch concentration and temperature 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 0.931. This means that this model fitted well with the experimental data. The R^2 value implies that the sample variation of 93.1% 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 4 indicates that the Linear and quadratic terms in second order polynomial Model equation (2) 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.

A. Localization of optimum condition for glucose production

The Contour plot describing combined effect between pair of factors on hydrolysis of potato starch were given in figure 1 to 6 by keeping other two variables constant at their middle level. Fig.1 indicates that glucose yield as a function of temperature and time. It was observed that middle to high level of time (60 to 120 min) and middle level of temperature, the glucose concentration significantly high.

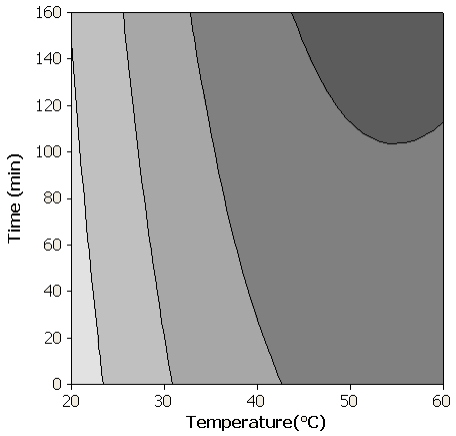

Fig. 1 Contour plot of the combined effect of temperature and time on glucose concentration

Fig. 2 explains that the glucose yield interaction effect of starch concentration and temperature. It indicates that the glucose concentration maximum at middle level of Starch concentration and Temperature. Fig.3 shows relation between starch concentration and time with the glucose yield. Glucose yield increases at middle level of starch concentration and a linear increase was observed with increase in low to high level of time. Relative effect of starch concentration and enzyme concentration on glucose yield was explained in Fig.4. From the Contour plot, at the middle level of starch concentration and high level of enzyme concentration, maximum glucose yield was observed. Combined effect of Enzyme concentration and time was shown in Fig.5. A linear increase in glucose yield was observed on both variables with increase from low to high level. Fig.6 explains the interaction effect of enzyme concentration and temperature. It was observed that glucose yield was observed from low to high level of enzyme concentration whereas glucose yield was increased at middle and decreased at low and high level of temperature.

IV. CONCLUSION

The present study using the RSM with CCD enables to find the importance of factors at different levels. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to

optimize the process for glucose production. The results of optimum value of starch concentration, enzyme concentration, temperature and time were found to be 6% (w/v), 2% (w/v), 40°C and 80min. The maximum glucose yield at optimum condition was 2.34(mg/ml).

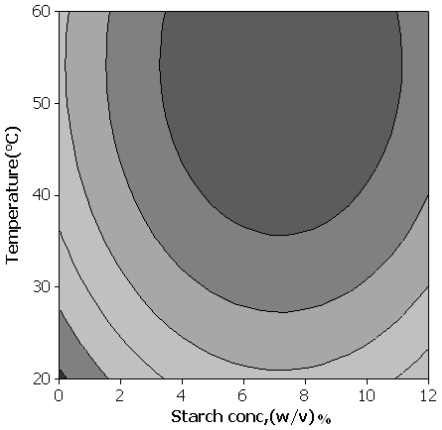

Fig. 2 Contour plot of the combined effect of temperature and starch concentration on glucose concentration

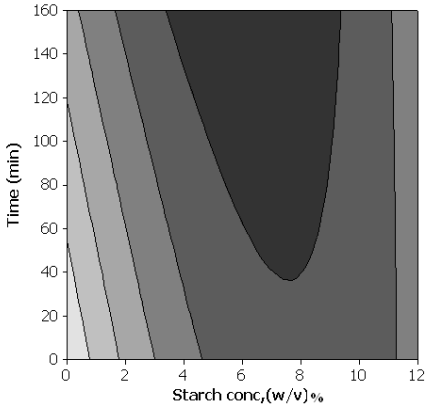

Fig. 3 Contour plot of the combined effect of time and starch concentration on glucose concentration

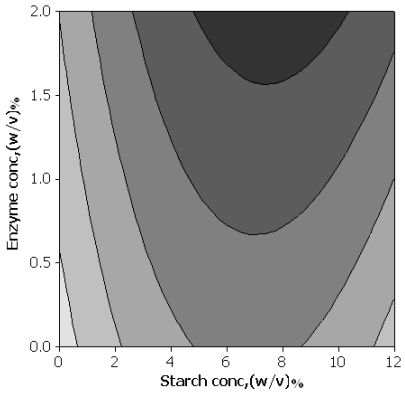

Fig. 4 Contour plot of the combined effect of starch concentration and enzyme concentration on glucose concentration

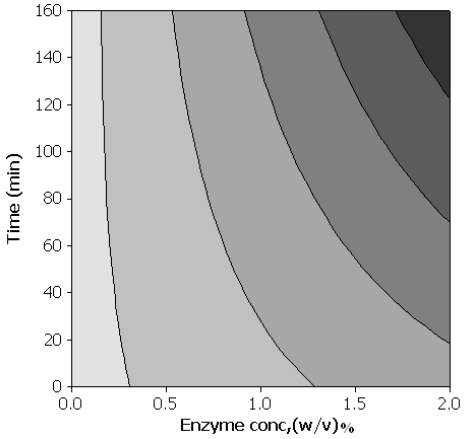

Fig. 5 Contour plot of the combined effect of time and enzyme concentration on glucose concentration

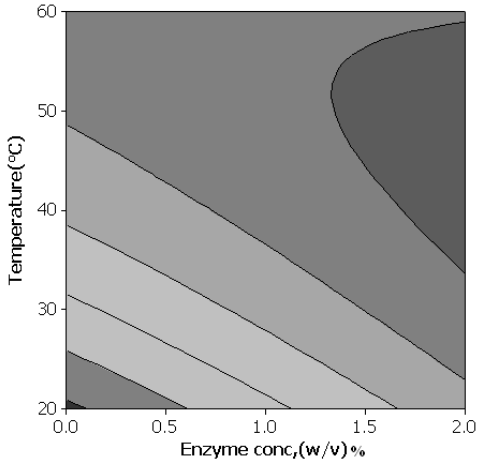

Fig. 6 Contour plot of the combined effect of temperature and enzyme concentration on glucose concentration

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