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Kinetic and Optimization Studies on Ethanol Production from Corn Flour

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Abstract**—**Studies on Simultaneous Saccharification and Fermentation (SSF) of corn flour, a major agricultural product as the substrate using starch digesting glucoamylase enzyme derived from *Aspergillus niger* and non starch digesting and sugar fermenting *Saccharomyces cerevisiae* in a batch fermentation. Experiments based on Central Composite Design (CCD) were conducted to study the effect of substrate concentration, pH, temperature, enzyme concentration on Ethanol Concentration and the above parameters were optimized using Response Surface Methodology (RSM). The optimum values of substrate concentration, pH, temperature and enzyme concentration were found to be 160 g/l, 5.5, 30°C and 50 IU respectively. The effect of inoculums age on ethanol concentration was also investigated. The corn flour solution equivalent to 16% initial starch concentration gave the highest ethanol concentration of 63.04 g/l after 48 h of fermentation at optimum conditions of pH and temperature. Monod model and Logistic model were used for growth kinetics and Leudeking – Piret model was used for product formation kinetics.

Keywords**—**Simultaneous Saccharification and Fermentation (SSF), Corn Starch, Ethanol, Logisitic Model.

I. INTRODUCTION

THE interest in biotechnology-based production of fuels tends to augment with the concern about exhaustion of fossil fuels and the increase in their price [1]. The use of petrol blended with20–24% ethanol is a standard practice in Brazil. Therefore, it is highly desirable for a country like India to use ethanol–petrol blend as transportation fuel to save valuable foreign exchange in importing crude oil as well as in reducing the environmental pollution caused by the vehicular emission. The most critical element for the success of bioethanol technology is the availability of celluloses at a nominal cost. Major R&D effort is required to produce cellulase with high yield and productivity [2,3]. Alternatively, thermo-tolerant high activity liquefying and saccharifying enzymes (a-amylase and glucoamylase) would be required for the development of cost-effective starch-based ethanol production in India [4, 5]. For the last two decades, ethanol production by the yeast *Sacchoromyces cerevisiae* has been studied extensively [6]. *S.cerevisiae* is capable of metabolizing few types of sugar such as glucose, fructose and sucrose [7,8]. Corn starch, an agricultural product, is a cheap substrate that is easily available in tropical countries like India. There are few reports available on fermentation of Corn starch hydrolysate by *S. cerevisiae*. Ethanol production from Corn starch requires the use of amylase and glucoamylase for the pretreatment of Corn starch before fermentation [9]. To reduce the fermentation time and to avoid a separate saccharification step, the substrate liquefied corn starch was fermented to ethanol by Simultaneous Saccharification and Fermentation (SSF) using glucoamylase and free cells of *S. cerevisiae* in a same fermentor. The use of SSF process is s highly desirable to achieve higher ethanol productivity.

The present study is aimed at optimization of the process variables affecting the ethanol production by simultaneous Saccharification and fermentation of liquefied corn starch solution to ethanol using glucoamylase which hydrolyses liquefied products to glucose and *S.cerevisiae* MTCC 463 which is non amylolytic but efficiently ferments glucose to ethanol. Model development for microbial growth kinetics and product formation kinetics is essential since their operation involves microbial growth under constantly changing conditions, with impact on process kinetics and performance.

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II. MATERIAL S AND METHODS

A. Materials

Corn flour, *Zea mays* L was obtained from a flour mill and stored in an air tight container. The proximal composition of corn flour in g/100 g of corn flour is found to be protein – 8.8, oils - 4.4, sugars -1.7, fiber -8.5, ash -1.7 and starch – 74.9.

B. Microorganisms and Culture Conditions

S. cerevisiae MTCC 463 procured from IMTECH, Chandigarh, INDIA was maintained in YMP agar medium with a composition of yeast extract 3.0 g/l, malt extract 3.0 g/l, peptone 5.0 g/l and agar 20 g/l at a pH of 5.5 and 28°C.

C. Media composition

The growth medium used for preparing *S. cerevisiae* contained in grams per 100 ml Glucose, 5; peptone, 0.5; yeast extract, 0.5; potassium dihydrogen phosphate, 0.1.The fermentation medium used for ethanol production from corn flour was identical to growth medium except that starch concentration of pretreated corn starch solution was varied from 5 to 20 g per 100 ml in different experiments. The Corn flour was pretreated with fungal amylase to liquefy the starch

present in it. The pretreated solution was filtered and the supernatant was analyzed for the reducing sugar concentration. The amount of starch present in the sample was then analyzed by phenol sulphuric acid method.

D. Pretreatment

A 25% (w/v) solution of corn flour was gelatinized in an autoclave at a pressure of 15 psi for one hour. The solution was cooled and pretreated using fungal α-amylase enzyme "Diastase" obtained from Hi media laboratories with enzyme activity of 1300 IU/g .The pretreatment was carried out in an Applikon fermentor at controlled conditions of pH 6 and temperature 70 °C for two hours using 2 g of Diastase enzyme to liquefy the starch present in the flour.

E. Simultaneous Saccharification and Fermentation (SSF)

Ethanol production by co-culture of mold and yeast was carried out using known activity of amyloglucosidase enzyme procured from Sigma Chemicals, U.S.A and 24 h-old slants of *S. cerevisiae* simultaneously. Fifty ml of 24 h old *S.cervisiae* cell culture containing $20x10^6$ cells were inoculated to 1000 ml of pretreated corn flour solution of known starch concentration in Applikon fermentor equipped with controllers for pH, temperature, agitation speed. The amyloglucosidase enzyme of known activity was added to the fermentation medium. Samples were withdrawn for every twelve hours, centrifuged in a variable speed research centrifuge at 5000 rpm, and the supernatants were analyzed for glucose and ethanol concentrations. Fermentations were carried out at various controlled temperatures, pH, Enzyme Concentration and substrate concentration as detailed in Section 2.6.

F. Experimental design and Statistical Analysis

Optimization of process parameters in the pretreatment of corn flour using fungal α-amylase was studied using CCD experiments. Substrate concentration (X_1, g/l), pH (X_2), temperature (X_3, °C) and glucoamylase enzyme concentration (X_4, IU) were chosen as the independent variables and is shown in Table 1. Ethanol concentration (Y, g/l) was used as the dependent output variable.

$$x_i = \frac{X_i - X_c}{\Delta x_i} \quad i = 1,2,3,4. \qquad (1)$$

The variables X_i were coded as x_i as per the equation (1) in which x_i is the dimensionless value of an independent variable, X_i the real value of the independent variable, X_c the real value of the independent variable at central point and Δx_i is the step change of variable i . The true values of the variables are also given in Table 1. A 2^4 factorial Central Composite experimental Design, with eight axial points and six replications at the centre points leading to a total number of 31 experiments was employed for the optimization of parameters and given in Table 2. The second degree polynomial equation (2) was solved using MINITAB 14 version statistical package.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 \qquad (2)$$

Where Y is the predicted response (Ethanol conc, g/l) x_1, x_2, x_3 and x_4 are the coded levels of the independent variables, b_0 the offset term, b_1 ,b_2, b_3, and b_4 the linear effects, b_{11}, b_{22}, b_{33} and b_{44} the quadratic effects and b_{12}, b_{13} ,b_{14}, b_{23}, b_{24} and b_{34} are the interaction effects. If the curve shape of the response surface plot is elliptical or circular then it is presumed that the interaction between the variables is most significant.

G. Cellmass and Chemical Analysis

The cell biomass was determined by harvesting cells by centrifuging at 5000 rpm, drying them at 70°C under vacuum to a constant weight and expressing the dry weight as grams per liter of growth medium. The corn flour sample and the fermentation medium were analyzed for starch by phenol sulphuric acid method and reducing sugar concentration was analyzed by dinitro salicylic acid (DNS) method [10] using Bio spectrophotometer (ELICO BL 198).

The extra cellular amylolytic activity was determined by measuring the reducing sugar released by 1%starch solution. One unit of amylolytic activity is defined as the amount of enzyme in 1 ml that liberates one micromole of reducing sugar in 3 min. Ethanol concentration in the fermented broth was determined using NUCON 5765 Gas Chromatography (GC) with a flame ionization detector and Chromatorpak 20Mcolumn (2m x 0.3cm) in which Nitrogen at 2 kg/cm^2 was used as the carrier gas. The oven temperature was maintained at 80°C. The injector and detector temperature was maintained at 200°C.

III. RESULTS AND DISCUSSION

A. Optimization of process variables on ethanol production

The factors affecting the Simultaneous saccharification and fermentation of Corn flour with Glucoamylase enzyme and S. *cerevisiae* culture was studied using CCD experiments. The substrate concentration (X_1, g/l), the pH (X_2), the temperature (X_3,°C) and the Glucoamylase enzyme concentration (X_4, IU) were chosen as the independent variables as shown in Table 1. Ethanol Concentration (Y) was chosen as the dependent output variable. Thirty one experiments based on the CCD were carried out with different combinations of variables and the results were presented in Table 2. The data obtained from the four level central composite design matrix were used to develop models in which each dependent variable (Ethanol Concentration, Y) was obtained as the sum of the contributions of the independent variable through second order polynomial equation and interaction terms. The actual ethanol concentration obtained in the experiments and the yields predicted by the model equation (2) are given in Table 3.

It showed that the regression coefficients of all the linear term and all quadratic coefficients of X_1, X_2, X_3 and X_4 were significant at < 1% level. The individual effect of all the four parameters studied, quadratic effects and interaction effects between the dependent variables were found to be significant from the response surface plots shown in Figs.1 to 3. The clear elliptical shape of the curve shown in Figs. 1 to 3

indicates the interaction effect between all the four independent variables were significant. Hence optimum combinations of substrate concentration, pH, pretreatment temperature, with the enzyme concentration play a major role in order to get maximum bioconversion of corn flour to ethanol. The ANOVA result of quadratic regression model for Y is described in Table 4. ANOVA of the regression model for Y demonstrated that the model was significant due to an *F*-value of and a very low probability value ($P < 0.001$). The *P*-values are used as a tool to check the significance of each of the coefficients, which in turn indicate the pattern of the interactions between the variables. Smaller value of *P* then it was more significant to the corresponding coefficient. Table 3 also showed that the experimental yields fitted the second order polynomial equation well as indicated by high R^2 values (0.992).

The orientation of the principal axes of the contour plots between the variables substrate concentration and temperature, substrate concentration and pH, Substrate Concentration and enzyme concentration pH and temperature, pH and enzyme concentration and temperature and enzyme concentration indicated that the mutual interactions between these set of variables had a significant effect on the ethanol Concentration. The values of P less than 0.005 in Table 3 also indicate the significance of interaction effects of all the four chosen independent variables. Based on the model, the optimal working conditions were obtained to attain high percentage conversion of starch. The optimum values of the parameters X_1, X_2, X_3 and X_4 were found to be 160 g/l, 5.5, 30°C and 50 IU respectively and were obtained by solving the regression equation (2) using the experimental data with square MATLAB version 7.0.

B. Effect of inoculum age on ethanol production

The growth of *S. cerevisiae* and simultaneous production of ethanol mainly depends on the production of glucose from Corn flour as a substrate. The inoculums age of *S. cerevisiae* was varied using 24 h old slant, 48 h old slant and 72 h old slant to study the effect of inoculation age on the ethanol concentration. Figure 4 shows the effect of inoculation age on the ethanol concentration. The 24 h old slant of *S. cerevisiae* gave a higher yield of ethanol than the 48 h old slant. Further increase in inoculums age gave lesser yield of ethanol than 24 h old slant. Hence it is recommended to use 24 h old slant culture of *S. cerevisiae* for efficient and economical production of ethanol.

C. Kinetics and Modeling

Monod model (growth)

The relation between the specific growth rate (μ) of a population of microorganisms and the substrate concentration (S) is very useful in the design of fermentor. The microbial growth kinetics was proposed by Monod (1942) and was used by most researchers for similar fermentation [11, 12]. The Monod model introduced the concept of growth limiting substrate and is given by equation (3).

$$\mu = \mu_{\max} \frac{S}{K_s + S} \quad (3)$$

TABLE I
CODES AND ACTUAL LEVELS OF THE INDEPENDENT VARIABLES FOR DESIGN OF EXPERIMENT

Independent variables	Symbols	Coded levels				
		– 2	– 1	0	+1	+2
Substrate conc. (g/l)	C	40	80	120	160	200
pH	P	4.5	5.0	5.5	6.0	6.5
Temp (°C)	T	28	30	32	34	36
Enzyme conc. (IU)	E	10	20	30	40	50

The maximum specific growth rate and substrate saturation constant were found to be 0.26 (h^{-1}), 14 g/l respectively using the Monod model. The model predicts very closely the microbial growth kinetics in ethanol production using co-culture fermentation.

TABLE II
FIVE LEVEL CCD AND THE EXPERIMENTAL RESPONSES OF DEPENDENT VARIABLE, Y

Run #	Substrate conc. (g/l) (A)	pH (B)	Temp. (°C) (C)	Enzyme conc. (IU) (D)	Ethanol conc.(g/l), Y	
					Exp	Pred
1	-1(80)	-1(5)	1(34)	1(40)	31.946	32.019
2	0(120)	0(5.5)	0(32)	2(50)	49.049	48.311
3	0(120)	0(5.5)	2(36)	0(30)	33.581	33.887
4	-1(80)	-1(5)	-1(30)	-1(20)	24.214	23.611
5	2(200)	0(5.5)	0(32)	0(30)	43.085	43.391
6	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
7	1(160)	1(6)	-1(30)	1(40)	42.274	42.569
8	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
9	1(160)	-1(5)	-1(30)	-1(20)	46.206	44.913
10	0(120)	-2(4.5)	0(32)	0(30)	38.67	39.573
11	-1(80)	1(6)	-1(30)	1(40)	36.166	35.619
12	1(160)	-1(5)	1(34)	1(40)	56.658	56.041
13	-1(80)	1(6)	1(34)	1(40)	25.412	25.707
14	1(160)	-1(5)	1(34)	-1(20)	39.78	40.405
15	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
16	1(160)	-1(5)	-1(30)	1(40)	63.048	63.121
17	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
18	1(160)	1(6)	-1(60)	-1(20)	30.136	30.137
19	-1(80)	1(6)	1(34)	-1(20)	21.606	21.607
20	0(120)	2(6.5)	0(32)	0(30)	18.463	18.485
21	0(120)	0(5.5)	-2(28)	0(30)	47.693	48.307
22	0(120)	0(5.5)	0(32)	-2(10)	24.34	26.003
23	1(160)	1(6)	1(34)	1(40)	28.94	29.617
24	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
25	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
26	0(120)	0(5.5)	0(32)	0(30)	49.037	49.037
27	-1(80)	1(6)	-1(30)	-1(20)	29.328	28.947
28	-1(80)	-1(5)	-1(30)	1(40)	35.756	36.059
29	-1(80)	-1(5)	1(34)	-1(20)	23.436	22.143
30	1(160)	1(6)	1(34)	-1(20)	21.06	19.757
31	-2(40)	0(5.5)	0(32)	0(30)	17.565	18.179

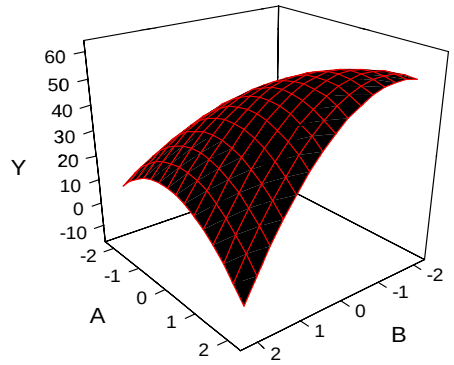

Fig. 1 Contour plot for the effect of substrate conc. (A) versus initial pH (B) on ethanol production

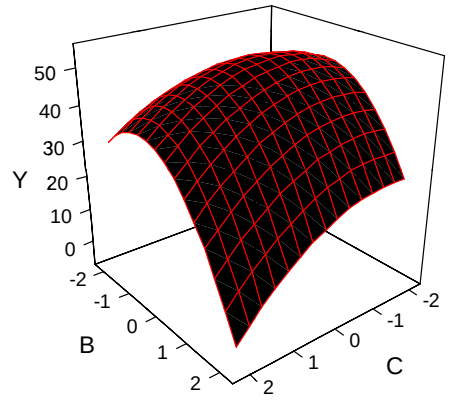

Fig. 2 Contour plot for the effect of initial pH (B) versus incubation temperature (C) on ethanol production

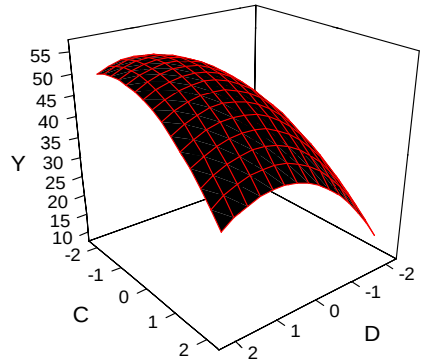

Fig. 3 Contour plot for the effect of incubation temperature (C) versus enzyme conc. (D) on ethanol production

Logistic model (growth)

The Logistic model for growth kinetics is shown in equation (4) and its integrated form in equation (5)

$$x = \frac{x_0 e^{kt}}{1 - \beta x_0 (1 - e^{kt})} \tag{4}$$

where, k – rate constant (h^{-1}), $\beta = 1/x_s$ (g of product /g of biomass - h)

$$\ln\left[\frac{x(t)/x_0}{1 - x(t)/x_o}\right] = kt - \ln\left[\frac{x_s - 1}{x_0}\right] \tag{5}$$

From the above linear plot the value of rate constant k was found to be 0.152 h^{-1}.

where, $\beta = (dp/dt)_{stationary} / x_s$, $(dp/dt)_{stationary}$ = 0.062(g of product/g of biomass - h)

Leudeking-Piret model (product formation)

The Leudeking – Piret kinetic model for product formation is given in equation (6) was found to fit the experimental data and the value of α and β were found to be 2.67 g of product/g of biomass and 0.062 g of product/g of biomass -h respectively.

$$p(t) - p_0 - \beta\left(\frac{x_s}{k}\right)\left[1 - \frac{x_0}{x_s}\left(1 - e^{kt}\right)\right] = \alpha\left[x(t) - x_0\right] \tag{6}$$

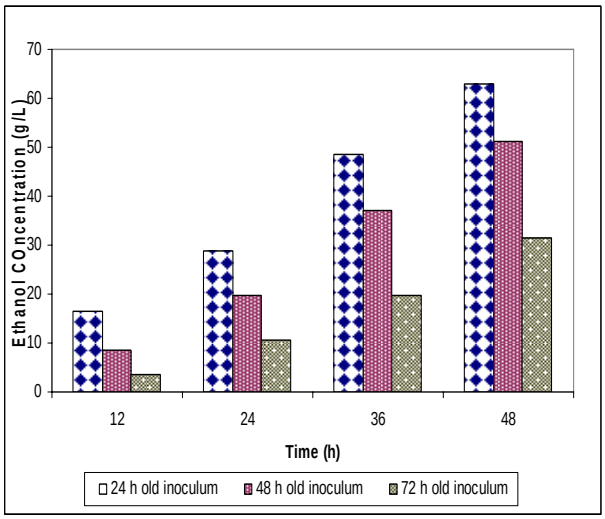

Fig. 4 Effect of inoculum age on ethanol production

IV. Conclusion

The ethanol production was studied using corn flour containing a high starch content of 20.9 % (w/w). SSF technique using glucoamylse enzyme derived from *A.niger* and non starch digesting, sugar fermenting *S.cerevisiae* was employed. The pretreatment of substrate using pure fungal alpha amylase was carried out before SSF to extract the starch from the corn flour. The parameters concerning SSF process namely, the substrate concentration, the pH, the temperature and the enzyme concentration were optimized using CCD and RSM. The optimum values of substrate concentration, the pH, the temperature and the enzyme concentration were found to be 160 g/l, 5.5, 30°C and 50 IU respectively. The maximum ethanol yield of 63.04 g/l was obtained at the optimum conditions of SSF. Monod model and Leudeking – Piret model were found to represent closely

the experimental data of growth kinetics and product formation kinetics respectively.

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