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# Optimization of C/N ratio of the medium and Fermentation conditions of Ethanol Production from Tapioca Starch using Co – Culture of *Aspergillus niger* and *Sachormyces cerevisiae*

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**Abstract:** Studies on Co-Culture fermentation of tapioca flour as the substrate symbiotic strains of starch digesting *Aspergillus niger* and non starch digesting and sugar fermenting *Saccharomyces Cerevisaie* in a batch fermentation. The effect of Carbon to Nitrogen (C/N) ratio of the fermentation medium on ethanol concentration and biomass was investigated. The optimum C/N ratio of the fermentation medium was found to be 35.2.which gave a maximum ethanol concentration of 8.85 g/l. Experiments based on Central Composite Design (CCD) were conducted to study the effect of pH, temperature and substrate concentration on ethanol yield from pretreated tapioca flour and the above parameters were optimized using response surface methodology (RSM). The optimum values of pH, temperature and substrate concentration were found to be 5.5, 30°C and 60 g/l respectively. The tapioca flour solution equivalent to 6% initial starch concentration gave the highest ethanol concentration of 8.9 g/l after 48 h of fermentation at optimum conditions of pH and temperature. Logistic model was used for growth kinetics and Leudeking – Piret model was used for product formation kinetics.

**Key words:** Co-culture fermentation; C/N ratio; Central Composite Design (CCD); Response Surface Methodology (RSM); Ethanol; Logistic model.

## 1. Introduction

Bio ethanol has stimulated worldwide interest due to its utilization as an alternative fuel source and is produced from renewable cheap agricultural resources[1]. High energy prices, increasing energy imports concerns about petroleum supplies, and greater recognition of the environmental consequences of fossil fuels have driven interest in bio fuels.[2,3] A low cost of feedstock is a very important factor in establishing a cost effective technology.[4, 5] Utilization of starch and cellulosic substrates for ethanol production are now preferred for economic reasons[6]. The starch substrates include cereal grains such as corn, wheat and starch root plants like cassava, the cost of which various according to crop yield and their use for animal or human consumption.[7] Tapioca starch is an agricultural material abundantly produced in India and other tropical countries[8].

Simultaneous Saccharification and Fermentation (SSF) of starch with an amylolytic mold and yeast is an efficient and economical method for ethanol production due to lesser equipment cost. [9, 10] Commercial enzyme glucoamylase is used for sachharification and represent a significant expense in the production process[11]. This study aims at eliminating the enzymatic saccharification step by using symbiotic Co–culture of amylolytic and sugar fermenting organisms[12].

Response Surface Methodology (RSM) is an important statistical technique employed for multiple regression analysis by using quantitative experimental data obtained from properly designed experiments using Central Composite Design (CCD).[13]

The Response Surface is a two-dimensional graphic representation to study the individual, interactive and cumulative effects of the variables. The authors reports

the application of the RSM using CCD experiments to develop a mathematical correlation between the pH, temperature and substrate concentration in Co-Culture fermentation of tapioca flour to ethanol concentration.

The mathematical models play an important role in rational design and optimization of biochemical process[14, 15]. It is difficult to obtain an accurate model for biochemical process such as ethanol production by co-culture method due to the inherent complexity[16].

The present study is aimed at optimization of the process variables affecting the ethanol concentration namely pH, temperature and starch concentration by RSM technique, optimization of the Carbon to Nitrogen ratio in simultaneous Saccharification and fermentation of alpha amylase treated tapioca flour solution to ethanol using co-culture of *Aspergillus niger* (MTCC 1349) which hydrolyses liquefied products to glucose and *Sachharomyces cerevisiae (*MTCC 171) which is non amylotic but efficiently ferments glucose to ethanol and model development for microbial growth kinetics and product formation kinetics.

## 2. Materials and Methods

### 2.1 Material

Tapioca flour was obtained from a flour mill and was dried and stored in an air tight container. The composition of tapioca flour in g/100 g of tapioca flour is found to be sugars-5.1, Fiber – 1.1, Protein – 1.1, starch – 22.9 and remaining moisture.

### 2.2 Microorganisms

The strain of *A. niger* (MTCC 1349) and ethanol producing thermo tolerant *K. marxianus* (MTCC1389) were obtained from IMTECH, Chandigarh. *A. niger* was maintained in a Potato Dextrose Agar (PDA) medium with a composition of potato 200 g/l dextrose 40 g/l and agar 20 g/l at a pH of 5.5 and 28°C. *S. cerevisiae* 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 30°C.

### 2.3 Media

The growth medium used for preparing *A. niger* inoculum contained in grams per 100 ml. Starch, 2; peptone, 0.5; yeast extract, 0.5; magnesium chloride, 0.1: ammonium phosphate, 0.1; and ferrous sulphate, 0.01.

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 tapioca flour was identical to growth medium except that starch concentration of pretreated tapioca solution was varied from 2 to 10 g per 100 ml in different experiments. The tapioca flour was pretreated with fungal amylase to extract 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 calculated by using the phenol sulfuric acid method.

### 2.4 Pretreatment of Tapioca Flour

Tapioca flour of 2.0% (w/v) 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 obtained from Hi media laboratories for an hour. The temperature and pH are maintained at 60 °C in a constant temperature water bath and 6 using phosphate buffer respectively.

### 2.5 Co-Culture Fermentation of Tapioca Flour

Ethanol production by co-culture of mold and yeast was carried out using cells from 72 h-old slants of *A. niger* and 24 h-old slants of *S. cerevisiae*. These cells were inoculated separately into flasks of 50 ml growth medium containing 2% starch. These flasks were incubated under shaking condition of 150 rpm, for 72h and 48 h at 30 °C respectively. 5 ml of the cell suspension of *A. niger* containing 1.21 x $10^8$ cells and 5 ml of *S. cerevisiae* cell culture containing $2x10^5$ cells were inoculated to 200 ml of pretreated tapioca flour solution with different starch concentrations. The fermentation was carried out for a period of 48 h. 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.

### 2.6 Experimental Design and Optimization

Optimization of process parameters in ethanol production from tapioca flour using Co-Culture fermentation was studied using CCD experiments. pH ($X_1$), temperature ($X_2$, °C) and substrate concentration ($X_3$, g/l) were chosen as the independent variables and is shown in Table 1. Ethanol yield (Y, g/l) was used as the dependent output variable.

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

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 $\Delta x_i$ is the step change of variable $i$ . The true values of the variables are also given in Table 1.

A $2^3$factorial Central Composite experimental Design, with six axial points and six replications at the centre points leading to a total number of 20 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.

$$Y=b_0+b_1x_1+b_2x_2+b_3x_3+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2+b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3 \quad \text{---------- (2)}$$

Where Y is the predicted response (Ethanol Yield, g/l ) $x_1$, $x_2$ and $x_3$ are the coded levels of the independent variables, $b_0$ the offset term, $b_1$ ,$b_2$ and $b_3$ the linear effects, $b_{11}$, $b_{22}$ and $b_{33}$ the quadratic effects and $b_{12}$, $b_{13}$ and $b_{23}$ 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.

**2.7 Cell Mass and 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 wheat bran flour sample was analyzed for starch by phenol sulphuric acid method and reducing sugar concentration was analyzed by di nitro salicylic acid (DNS) method [17]using Bio spectrophotometer (ELICO BL 198).

**2.8 Ethanol Estimation**

Ethanol concentration in the fermented broth was determined using NUCON 5765 Gas Chromatography (GC) with a flame ionization detector and Poropak Q column (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.

**3. Results and Discussion**

**3.1 Effect of C/N Ratio on Ethanol Production**

C/N ratio of fermentation medium plays a vital role in production of ethanol by SSF process. The C/N ratio of the fermentation medium was varied from 3.5 to 35.2 using nitrogen sources namely yeast extract and peptone and the medium composition are given in Table 1. The results of experiments are also given in Table 1. The cell mass was found to increase with decrease in C/N ratio and maximum yield of 15.3 g/l was obtained at C/N ratio of 35.2. The results indicate that a higher C/N ratio gave maximum yield of ethanol of 8.85 g/l. The ethanol concentration in the fermentation medium was found to be decrease drastically with increasing nitrogen concentration. A very low yield of 0.55 g/l ethanol was obtained, when C/N ratio was 3.5. Hence an optimum C/N ratio of 35.2 must be used in fermentation medium to maximize the ethanol concentration.

**3.2 Optimization of pH, Temperature and Starch concentration of Co-Culture Fermentation using RSM**

The factors affecting the Ethanol Yield from pretreated tapioca flour using CO-Culture of *A. niger* and *S.cerevisiae* was studied using CCD experiments. The pH ($X_1$), the temperature ($X_2$, °C) and the starch concentration ($X_3$, g/l) were chosen as the independent variables as shown in Table 2. Ethanol Yield (Y, g/l) was chosen as the dependent output variable. Twenty experiments based on the CCD were carried out with different combinations of variables and the results were presented in Table 3. The data obtained from the three level central composite design matrix were used to develop models in which each dependent variable (Ethanol Yield, Y) was obtained as the sum of the contributions of the independent variable through second order polynomial equation and interaction terms. The actual yields of ethanol 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 $X_1$, $X_2$ and $X_3$ were significant at < 1% level. The individual effect of all the four parameters studied, quadratic effects and interaction effects between the temperature and substrate concentration were found to be significant from the response surface plots shown in Figs. 1, 2 & 3The clear elliptical shape of the curve shown in Figs. 2 indicates that the interaction effect between the substrate concentration ($X_3$) and temperature ($X_2$), is significant with a P value of 0.024.Hence an optimum combination of substrate concentration and temperature is a must in order to get maximum bioconversion of tapioca starch 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 29.01 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 4 also showed that the experimental yields fitted the second order polynomial equation well as indicated by high $R^2$ values (0.966).

The response surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and effects of interactions of each independent variable, on the response variables. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. From the response surface plots, the optimal values of the independent variables could be observed and the interaction between each independent variable pair can de described. The orientation of the principal axes of the contour plots between the variables substrate concentration and temperature, pH and temperature, and temperature and enzyme concentration indicated that the mutual interactions between these set of variables had a significant effect on the percentage conversion of starch.

The isoresponse contour plots of RSM as a function of two factors at a time, holding all other factors at fixed coded level (zero, for instance), are helpful for understanding both the main and the interaction effects of these two factors. The effect of varying levels of temperature and substrate concentration on the ethanol production, while other variable pH was fixed at central level, is shown in Fig. 1. When the other variable was kept constant, the interaction between the two variables (substrate concentration and temperature) showed that the ethanol yield was sensitive even when substrate concentration and temperature were subject to small alterations (Fig. 1). Under certain conditions a maximal contour (ethanol concentration of 8.9 g/l) could be determined, meaning that further change in temperature and pH would not increase the ethanol yield any further. The other pair of the independent variables pH and temperature showed similar effects while keeping the other independent variable, substrate concentration as constant at 60 g/l (Fig. 2).The contour plot for pH and substrate concentration on the yield of ethanol, where the variable temperature was kept constant at 30°C, showed that the ethanol yields were obtained in the middle level of the process variables (Fig. 3).

The results showed that as the values of process variables increased, the yield also increased but only up to the midpoint of range of variables and thereafter the yield decreased even though the values of variables increased. The ethanol yield was significantly affected by substrate concentration, pH and temperature

Based on the model, the optimal working conditions were obtained to attain high percentage conversion of starch. The optimum values of the parameters $X_1$, $X_2$, $X_3$ were found to be 5.5, 30°C and 60 g/l respectively and were obtained by solving the regression equation (2) using the experimental data with square MATLAB 7.0 version.

## 4. Modeling

### 4.1 Logistic Model

The Logistic model for growth kinetics [18] is shown in equation (4) and its integrated form in equation (4)

$$x = \frac{x_0 e^{kt}}{1 - \beta x_0 (1 - e^{kt})} \qquad \text{---------- (3)}$$

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

$$\ln\left[\frac{x(t)/x_0}{1 - x(t)/x_o}\right] = kt - \ln\left(\frac{x_s - 1}{x_0}\right) \qquad \text{---------- (4)}$$

From the above linear plot the value of rate constant k was found to be 0.0952 $h^{-1}$

Where, $\beta = \frac{\left(\frac{dp}{dt}\right)_{stationary}}{x_s}$, $(dp/dt)_{stationary} = 0.023$ (g of product/g of biomass - h)

### 4.2 Product Formation Kinetics

$$p(t) - p_0 - \beta\left(\frac{x_s}{k}\right)\left[1 - \frac{x_0}{x_s}\left(1 - e^{kt}\right)\right] = \alpha\left[x(t) - x_0\right] \qquad \text{---------- (5)}$$

The Leudeking – Piret kinetic model [19] for product formation is given in equation (5) was found to fit the experimental data and the value of $\alpha$ and $\beta$ were found to be 1.45 g of product/g of biomass and 0.023 g of product/g of biomass - h respectively.

**Table 1 :Effect of C/N ratio on ethanol yield and cell mass in CO-Culture fermentation of pretreated tapioca flour (6% w/v starch concentration) with experiments carried out at a pH of 5.5, temperature at 30 °C and 150 rpm**

| Nitrogen Concentration (g/l) | Nitrogen Concentration (g/l) | C/N ratio | Cell mass | Ethanol Yield |
|---|---|---|---|---|
| Peptone | Yeast extract | | g/l | g/l |
| 20 | 20 | 3.5 | 5.2 | 0.55 |
| 12.5 | 12.5 | 5.63 | 6.5 | 0.80 |
| 10.5 | 10.5 | 6.7 | 7.2 | 0.96 |
| 9 | 9 | 7.8 | 7.8 | 1.15 |
| 7.5 | 7.5 | 10 | 8.7 | 1.34 |
| 5 | 5 | 14.08 | 10.1 | 3.34 |
| 4 | 4 | 17.6 | 11.7 | 4.04 |
| 3 | 3 | 23.46 | 12.5 | 5.14 |
| 2 | 2 | 35.2 | 15.3 | 8.85 |

**Table 2: Codes and actual levels of the independent variables for design of experiment using CCD**

| Independent variables | Coded levels | | | | |
|---|---|---|---|---|---|
| | – 1.682 | –1 | 0 | 1 | 1.682 |
| pH (P) | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 |
| Temperature ( °C) | 26 | 28 | 30 | 32 | 34 |
| Substrate conc. (g/l) | 20 | 40 | 60 | 80 | 100 |

**Table 3.: Three level central composite design and the experimental responses of dependent variable Ethanol Yield, Y**

| Run order | pH | Temp. (°C) | Substrate conc.(g/l) | Ethanol Yield, Y | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 2 | 0(5.5) | 0(30) | 1.682(100) | 3.9 | 4.22 |
| 3 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 4 | 0(5.5) | -1.682(26) | 0(60) | 3.62 | 4.06 |
| 5 | 1(6.0) | -1(28) | 180) | 4.5 | 4.29 |
| 6 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 7 | 1.682(6.5) | 0(30) | 0(60) | 5.3 | 5.33 |
| 8 | -1(5.5) | 1(32) | -1(40) | 7.1 | 6.64 |
| 9 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 10 | 0(5.5) | 0(30) | -1.682(20) | 5.21 | 5.80 |
| 11 | 0(5.5) | 1.682(3.4) | 0(60) | 6.98 | 7.46 |
| 12 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 13 | 0(5.5) | 0(30) | 0(60) | 8.9 | 8.87 |
| 14 | 1(6.0) | 1(32) | -1(40) | 7.64 | 7.30 |
| 15 | -1(5.0) | 1(32) | 1(80) | 5.7 | 5.13 |

| | | | | | |
|---|---|---|---|---|---|
| 16 | 1(6.0) | 1(32) | 1(80) | 4.84 | 4.85 |
| 17 | -1(5.0) | -1(28) | -1(40) | 3.82 | 3.15 |
| 18 | -1.682(4.5) | 0(30) | 0(60) | 3.42 | 4.29 |
| 19 | 1(5.5) | -1(28) | -1(40) | 4.76 | 4.67 |
| 20 | -1(5.0) | -1(28) | 1(80) | 4.04 | 3.72 |

**Table 3.-: Results of regression analysis and corresponding *t* and p- value of second order polynomial model for optimization of ethanol production**

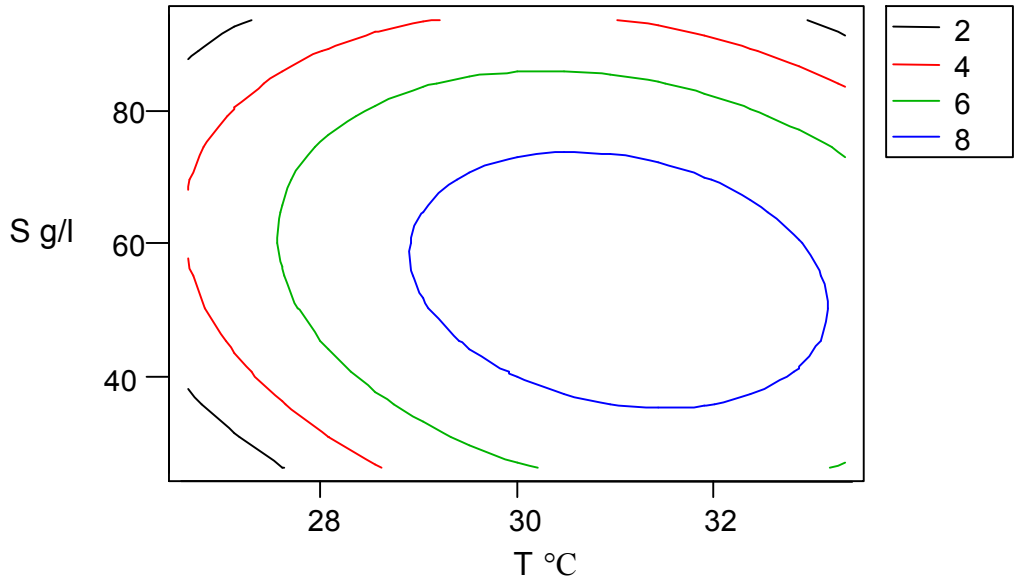
| Term constant | Regression coefficient | Std deviation | *t*- statistic | p-value |
|---|---|---|---|---|
| Constant | 8.874 | 0.2191 | 40.507 | 0.0001 |
| $(b_1)P^b$ | 0.311 | 0.1453 | 2.137 | 0.058 |
| $(b_2)T^c$ | 1.011 | 0.1453 | 6.958 | 0.0001 |
| $(b_3)S^d$ | -0.472 | 0.1453 | -3.246 | 0.009 |
| $(b_{11})$P*P | -1.433 | 0.1415 | -10.130 | 0.0001 |
| $(b_{22})$T*T | -6.340 | 0.1415 | -7. 782 | 0.0001 |
| $(b_{33})$S*S | -2.006 | 0.1415 | -9.643 | 0.0001 |
| $(b_{12})$P*T | -0.461 | 0.1899 | -1.132 | 0.284 |
| $(b_{13})$P*S | 0.262 | 0.1899 | -1.237 | 0.244 |
| $(b_{23})$T*S | -0.764 | 0.1899 | -2.738 | 0.021 |

[a]S – Substrate concentration (g/l)
[b]P – pH
[c]T – Temperature (°C)

**Table 4:Analysis of variance (ANOVA) for the fitted quadratic polynomial model for ethanol production**

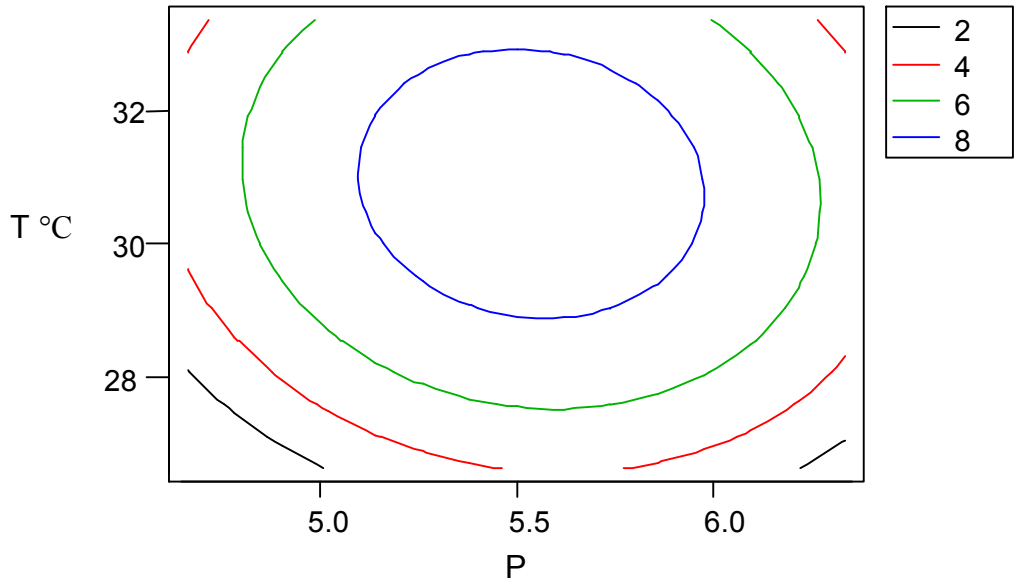
| Source | Sum of squares | Degrees of freedom (DF) | Mean square (MS) | *F*-value | p-value |
|---|---|---|---|---|---|
| Regression | 3572.36 | 9 | 9.244 | 32.04 | <0.001 |
| Linear | 1673.99 | 3 | 418.496 | 21.17 | <0.001 |
| Square | 1746.33 | 3 | 436.584 | 71.52 | <0.001 |
| Interaction | 152.04 | 3 | 25.34 | 3.44 | 0.060 |
| Residual Error | 140.74 | 10 | 8.796 | - | - |
| Lack-of-Fit | 140.74 | 5 | 14.074 | - | - |
| Pure Error | 0.0000 | 5 | 0.00000 | - | - |
| Total | 3713.09 | 19 | - | - | - |

$R^2 = 0.962$




Hold values: P: 5.5

**Fig. 1 Contour plot of substrate concentration (S) versus temperature (T) on ethanol yield (Y)**




Hold values: S: 60 g/l

**Fig.2 Contour plot of temperature (T) versus pH (P) on Ethanol Yield (Y)**

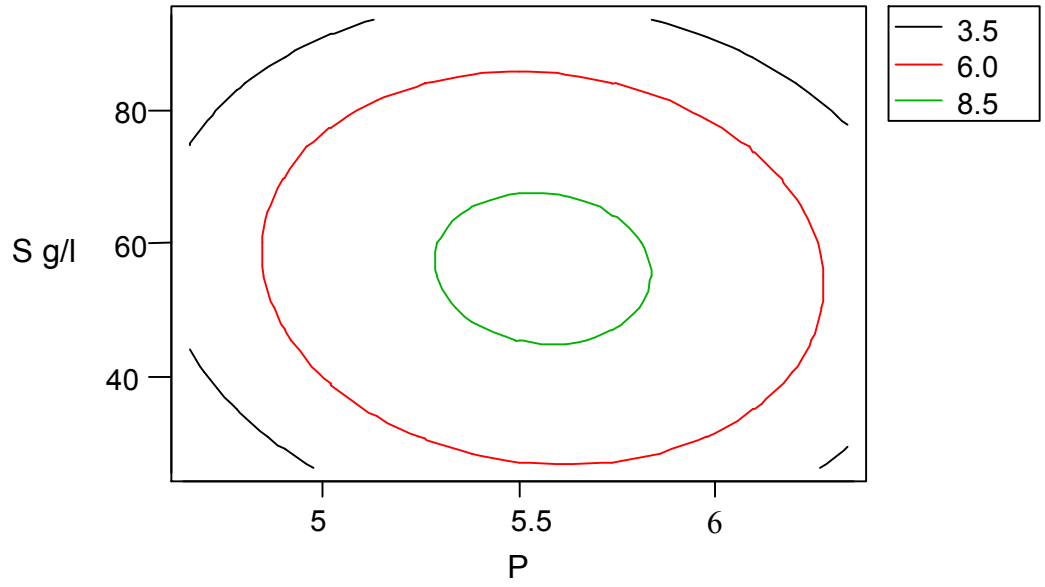



**Fig. 3 Contour plot of substrate concentration (S) versus pH (P) on ethanol yield (Y)**

## 5. Conclusion

The optimum C/N ratio of the fermentation medium was found to be 35.2 which gave a maximum ethanol concentration of 8.9 g/l. The optimum conditions for Co- Culture fermentation of tapioca flour were found to be the temperature 30°C, the initial pH 5.5 and the substrate concentration 60 g/l starch level using Response Surface Methodology. The maximum ethanol yield of 8.9 g/l was obtained at the optimum conditions of SSF. Logistic model and Leudeking – Piret model were found to represent closely the experimental data of growth kinetics and product formation kinetics respectively. The kinetic parameters of the models were evaluated.

## Nomenclature

α – kinetic parameter of Leudeking – Piret model (gP/gX)

β – kinetic parameter of Leudeking – Piret model (gP/gX-h)

$x_o$ – initial cell mass concentration (g/l)

$K_s$ – saturation constant (g/l)

S – substrate concentration (g/l)

$x_s$ – maximum stationary phase biomass concentration (g/l)

k – rate constant ($h^{-1}$)

t – time (h)

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