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Statistical Optimization of Process Variables for Direct Fermentation of 226 White Rose Tapioca Stem to Ethanol by *Fusarium oxysporum*

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Abstract**—**Direct fermentation of 226 white rose tapioca stem to ethanol by Fusarium oxysporum was studied in a batch reactor. Fermentation of ethanol can be achieved by sequential pretreatment using dilute acid and dilute alkali solutions using 100 mesh tapioca stem particles. The quantitative effects of substrate concentration, pH and temperature on ethanol concentration were optimized using a full factorial central composite design experiment. The optimum process conditions were then obtained using response surface methodology. The quadratic model indicated that substrate concentration of 33g/l, pH 5.52 and a temperature of 30.13ºC were found to be optimum for maximum ethanol concentration of 8.64g/l. The predicted optimum process conditions obtained using response surface methodology was verified through confirmatory experiments. Leudeking-piret model was used to study the product formation kinetics for the production of ethanol and the model parameters were evaluated using experimental data.

Keywords**—**Fusarium oxysporum, Lignocellulosic biomass, Product formation kinetics, Statistical experimental design

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I. Introduction

THE increasing gas prices and environmental concerns, in recent years, have becomes the driving force for developing alternative energy sources, especially fuel ethanol for automobiles [1]. Agricultural, agro-industrial and forestry lignocellulosic residues have potential to provide a more economical feed stock as a result of its widespread availability, sustainable production and low starting value [2]. Numerous systems are being studied as potential methods for obtaining ethanol from cellulose. Conventional methods applied for bioconversion of cellulose and hemi cellulose to ethanol, involve acid or enzyme hydrolysis of the biopolymers to soluble oligosaccharides followed by fermentation to ethanol. An alternative approach has been a direct process in which one or more microorganisms carry out simultaneous hydrolysis and fermentation in the same bioreactor [3]. Very few fungal species have the ability to convert cellulose directly to ethanol. Among them the strain of *Fusarium oxysporum* has been recently reported to ferment cellulose to ethanol with promising yields [4]. Besides cellulases, xylanases produced by *F.oxysporum* have been also characterized; more over it produces enough β-glucosidase activity to prevent cellobiose inhibition during hydrolysis [5], [6].

The possible variables that could be affecting the ethanol production were substrate concentration, pH and temperature. In order to identify the optimum conditions, 2^3 full factorial central composite design (CCD) using response surface methodology (RSM) was employed. The conventional method of optimization involves varying one parameter at a time and keeping the others constant. This often does not bring about the effect of interaction of various parameters as compared to factorial design. But the experiments conducted using the factorial designs, enable all factors to vary simultaneously. This helps in quantifying linear, square and interactive effects of the test variables. Another important advantage is that, the experimental designs could be changed progressively until a fitted model is found to describe the studied phenomenon [7], [8].

Response surface methodology is an empirical statistical technique employed for multiple regression analysis of quantitative data obtained from statistically designed experiments by solving the multivariate equations simultaneously. The graphical representation of these equations are called as response surfaces, could be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interaction between the test variables and their subsequent effect on the response [9], [10].

Lignocellulosic biomass such as Tapioca stem can be used only for direct burning. The conversion of tapioca stem to ethanol is more challenging due to the complex structure of the plant cell wall. Pre-treatment is required to alter the structural and chemical composition of lignocellulosic biomass to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars [11].

In the present study, sequential pre-treatments using dilute acid and dilute alkali solutions were performed to produce a cellulosic enriched material. The objective of this study was to identify the optimum process conditions for the selected operating variables namely substrate concentration, pH and temperature for the maximum production of ethanol using enriched 226 white rose tapioca stem. Kinetic process

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parameters have been determined to describe the product formation by *Fusarium oxysporum*.

II. MATERIALS AND METHODS

A. Materials

All the investigations were performed using 226 white rose tapioca stem as raw material, obtained from local farmers in Chidambaram, Tamilnadu, India.

B. Microorganisms and Culture conditions

The fungal culture *Fusarium oxysporum*(MTCC 284) was obtained from IMTECH, Chandigarh, India. The stock culture was maintained on potato sucrose agar medium with a composition of scrubbed and diced potatoes 200g/l, sucrose 20g/l and agar 20g/l at pH of 6.0 and 30°C. The production medium had the following composition per liter of distilled water: KH_2PO_4, 2g; $MgSO_4$, 0.3g; $CaCl_2$, 0.3g; peptone, 5g; yeast extract, 3g; malt extract, 3g; $FeSO_4.H_2O$, 0.05g; $ZnSO_4.4H_2O$, 0.014g; $MnSO_4.4H_2O$, 0.016g; $CoCl_2$, 2g; and substrate.

C. Physical pretreatment

After collection, the tapioca stems were crushed into small pieces and air-dried at 40°C in hot air oven. The dried stems were milled in a laboratory ball mill and screened through various mesh sizes namely 60, 100 and 150 respectively. The cellulose content of the raw tapioca stem was found to be 56.20% (dry basis). The effect of substrate size on ethanol concentration was carried out in 250 ml Erlenmeyer flasks with production medium by varying the substrate size with a concentration of 20g/l and an inoculum concentration of 2 %(v/v). The optimum mesh size which gives the maximum production of ethanol was used for further studies.

D. Sequential Pretreatment with dilute acid and dilute alkali

Ten grams samples of dried untreated tapioca stem of 100 mesh particles were suspended in 80 ml of 1.25% (w/v) H_2SO_4 solution in a 250 ml beaker at 120°C for 17 min. After reaction, the residues were separated by centrifugation and washed extensively with water until neutral pH and dried at 55°C. Acid treated samples were then suspended in 20 ml of 2% (w/v) NaOH aqueous solution in a 100 ml beaker at 120°C for 90 min. The residues were separated by filtration in 100% polyester cloth, washed with water to remove residual alkali and dried at 55°C. The cellulose content of untreated particles was determined consecutively.

E. Batch fermentation studies

Batch fermentations were carried out in 250 ml Erlenmeyer flasks with 1000 ml of production medium. The medium was distributed equally in ten sterile flasks, each containing various initial concentrations of substrate. 2% (v/v) of inoculum medium was transferred to each 100 ml production medium in sterile conditions. The flasks were gently agitated on a shaker with a constant shaking rate at 150 rpm. Samples were taken from the solution at regular time intervals for the analysis of cellulose concentration, biomass concentration and ethanol concentration. The cellulose content was measured by Anthrone reagent method [12] using Bio-Spectrophotometer (ELICO BL 198) at 630 nm. Ethanol concentration in the fermented broth was estimated using NUCON 5765 Gas Chromatography with a flame ionization detector. Biomass concentration was determined by centrifuging the samples at 5000 rpm. The settled biomass was collected and dried and expressing the dry weight as grams per liter of growth medium.

F. Experimental design and Statistical Analysis

The effects of various operating variables namely initial substrate concentration, pH and temperature on ethanol concentration was studied by central composite design and optimization using response surface methodology. For this study, 2^3 full factorial central composite design with six star points and six replicates at the centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure.

The experiments with five different substrate concentrations namely 10, 30, 50, 70 and 90 g/l, five different pH values of 5.0, 5.5, 6.0, 6.5 and 7.0 and five different temperatures of 26, 30, 34, 38 and 42°C were employed and varied simultaneously to cover the combinations of variables in the design. The range and the levels of the experimental variables investigated in this study were given in Table I. The chosen independent variables used in this experiment were coded according to equation (1).

$$x_i = X_i - X_o / \Delta x \tag{1}$$

where x_i = coded value of the i^{th} variable, X_i = uncoded value of the i^{th} test variable, and X_o = uncoded value of the i^{th} test variable at the center point.

The behaviour of the system is explained by the following empirical second-order polynomial model equation (2).

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \tag{2}$$

where Y = predicted response, β_o = offset term, β_i = coefficient of linear effect, β_{ii} = coefficient of square effect, and β_{ij} = coefficient of interaction effect.

TABLE I
RANGE AND LEVELS OF THE INDEPENDENT VARIABLES

Independent variable	Range and level				
	-α	-1	0	1	+ α
Substrate concentration(g/l),X_1	10	30	50	70	90
pH, X_2	5	5.5	6	6.5	7
Temperature(°C), X_3	26	30	34	38	42

The design package Minitab 14, a statistical program software, was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables

were obtained by solving the regression equation and also by analyzing the response surface contour plots.

G. Kinetics and Modelling

The simplest types of product formation kinetics arise when there is a simple stoichiometric connection between product formation and substrate utilization of cell growth. In such fermentation, especially those involving secondary metabolites, significant product formation does not occur during the log phase where product formation is proportional to the growth rate of cells. The product formation occurs late in the log phase or in the stationery phase. One such behavior is the Leudeking-piret kinetic model. This model combines both growth-associated and non-growth-associated models [13].

$$P(t) - P_o - \beta\left(\frac{X_s}{k}\right)\left[1 - \frac{X_o}{X_s}\left(1 - e^{Kt}\right)\right] = \alpha\left[X(t) - X_o\right] \quad (3)$$

where P(t) = product concentration at any time t (g/l), P_o = initial product concentration (g/l), X_s = the biomass concentration in stationery phase (g/l), X_o = initial biomass concentration (g/l), X(t) = biomass concentration at any time (g/l), and β, α & k = constants.

III. RESULTS AND DISCUSSION

A. Effect of Substrate size on the production of ethanol

From the experiment, the fermentation time was found to be 144h. Fig. 1 indicates the effect of substrate size of untreated ball milled tapioca stem particles on the concentration of ethanol. Maximum production of ethanol was obtained when the mesh size was 100 mesh particles which have the average diameter of 0.197 mm with a substrate concentration of 20 g/l in 8 days. Particles size and consequently surface area have been reported to be important factors in the hydrolysis of cellulose [14]. It was concluded that high ethanol yield and easy access of microorganisms to cellulose was achieved at 100 mesh particles.

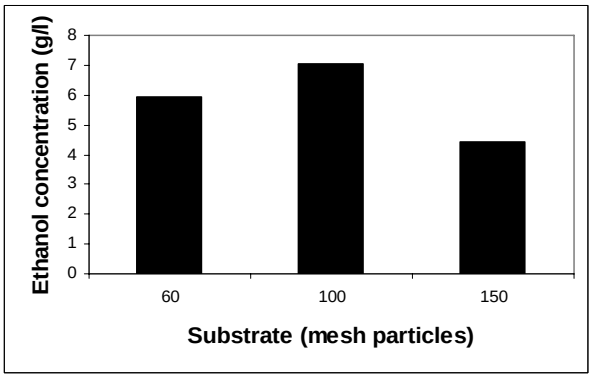

Fig. 1 Effect of substrate size on the production of ethanol

B. Effect of pretreatment on cellulose content for the production of ethanol

Fig. 2 shows the cellulose content (dry basis) for the untreated (UT), acid treated (AT) and acid-alkali treated (AAT) substrate for the production of ethanol. Acid pretreatment followed by alkali pretreatment under specific conditions gave 82.15% of cellulose content when compared with untreated material. It was concluded that, sequential pretreatment of tapioca stem leads the fractionation of the three components and opening of cellulose structure results in significant changes in specific surface area thereby improve the material digestibility and easy access for microbial attack.

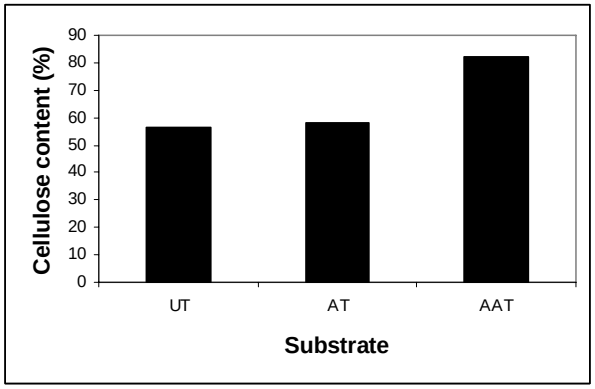

Fig. 2 Effect of pretreatment on cellulose content for the production of ethanol

C. Central Composite Design and Optimization using Response Surface Methodology

The coded and uncoded values of the independent variables along with observed responses in each case were given in Table II.

TABLE II
CCD MATRIX OF ORTHOGONAL AND REAL VALUES ALONG WITH OBSERVED RESPONSES

Exp. No.	Orthogonal & Real values			Ethanol concentration (g/l)
	X_1	X_2	X_3	
1	-1 (30)	1 (6.5)	1 (38)	6.70
2	1 (70)	-1 (5.5)	-1 (30)	7.00
3	1 (70)	1 (6.5)	1 (38)	6.80
4	1 (70)	-1 (5.5)	1 (38)	8.20
5	1 (70)	1 (6.5)	-1 (30)	7.30
6	-1 (30)	-1 (5.5)	-1 (30)	8.60
7	-1 (30)	1 (6.5)	-1 (30)	8.40
8	-1 (30)	-1 (5.5)	1 (38)	7.80
9	1.68 (90)	0 (6)	0 (34)	8.00
10	-1.68(10)	0 (6)	0 (34)	7.90
11	0 (50)	1.68 (7)	0 (34)	7.50

12	0 (50)	-1.68 (6)	0 (34)	7.20
13	0 (50)	0 (6)	1.68(42)	6.90
14	0 (50)	0 (6)	-1.68(26)	7.60
15	0 (50)	0 (6)	0 (34)	6.50
16	0 (50)	0 (6)	0 (34)	6.50
17	0 (50)	0 (6)	0 (34)	6.50
18	0 (50)	0 (6)	0 (34)	6.50
19	0 (50)	0 (6)	0 (34)	6.50
20	0 (50)	0 (6)	0 (34)	6.50

By applying multiple regression analysis on the experimental data, the following second order polynomial equation explains the ethanol production:

$$Y = 6.4993 - 0.1488X_1 - 0.1388X_2 - 0.2180X_3 + 0.5172X_1X_1 + 0.3051X_2X_2 + 0.2697\,X_3X_3 + 0.0250\,X_1X_2 + 0.4000X_1X_3 - 0.3250\,X_2X_3 \quad (4)$$

where X_1, X_2 and X_3 = coded values of the test variables namely substrate concentration, pH and temperature respectively.Multiple regression coefficient (R = 0.91) was estimated from the second-order polynomial Equation (4). The closer the value of R to 1 shows the better correlation between the observed and predicted values. The student t distribution and corresponding p values, along with the parameter estimate were given in Table III.

TABLE III
SIGNIFICANCE OF REGRESSION COEFFICIENTS

Model Term	Parameter estimate (Coefficients)	t	P
Constant	6.4993	53.354	0.000
X_1	-0.1488	-1.841	0.095
X_2	-0.1388	-1.717	0.117
X_3	-0.2180	-2.697	0.022
$X_1 * X_1$	0.5172	6.574	0.000
$X_2 * X_2$	0.3051	3.878	0.003
$X_3 * X_3$	0.2697	3.428	0.006
$X_1 * X_2$	0.0250	0.237	0.818
$X_1 * X_3$	0.4000	3.788	0.004
$X_2 * X_3$	-0.3250	-3.078	0.012

The coefficient for the substrate concentration (X_1) indicates that the ethanol concentration was more at lower substrate concentration. The decreased conversion may be due to insufficient amount of biomass used for fermentation and also probably due to inhibition of fermentation by the compounds resulting from sugar and lignin degradation [15], [16]. The effect of temperature was found to be highly significant (p= 0.022) on ethanol production. The squared effects of all the parameters were also found to be significant. The coefficient of the interaction terms of substrate concentration and temperature (p = 0.004) were found to be highly significant. The significance of each term in the second-order polynomial equation was validated by the statistical tests called the Analysis-of-variance (ANOVA) and the results were given in Table IV. ANOVA of the regression model was highly significant as was evident from the calculated F value (10.83) and a very low probability (p model>F=0.0001). It was observed that the coefficient for the squared effect was highly significant (p=0.0001) when compared with the individual and interactive effects.

TABLE IV
ANOVA SUMMARY OF QUADRATIC MODEL

Sources of variation	Sum of squares	Mean square	F	P
Regression	8.697	0.966	10.83	0.000
Linear	1.214	0.405	4.54	0.030
Square	5.353	1.784	20.00	0.000
Interaction	2.130	0.710	7.96	0.005
Residual error	0.892	0.089		
Total	9.589			

For an easy evaluation of the effect of different process parameters on the production of ethanol, response surfaces were drawn. Response surface was generated by plotting the response (Ethanol concentration) against any two independent variables at their respective '0' levels. Fig. 3, Fig. 4 and Fig. 5 shows the response surface plots against each of the independent variables while keeping the other variables at their '0' levels. The maximum predicted yield was indicated by the surface confined in the smallest curve of the response surface diagram.

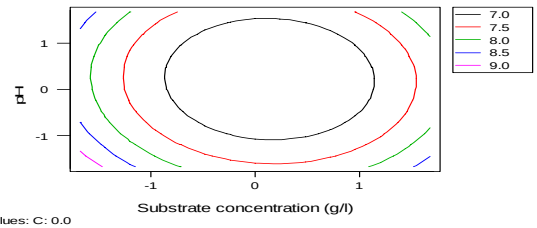

Fig. 3 Response surface contour plot for the interactive effect of substrate concentration and pH on the production of ethanol

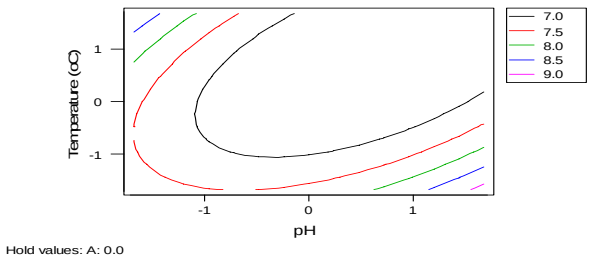

Fig. 4 Response surface contour plot for the interactive effect of pH and temperature on the production of ethanol

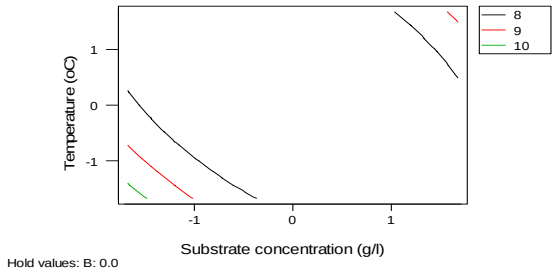

Fig. 5 Response surface contour plot for the interactive effect of substrate concentration and temperature on the production of ethanol

The second degree polynomial Equation (4) was solved by sequential quadratic programming using MATLAB 7.0. The optimum values of the test variables were first obtained in coded units and then converted to uncoded units for the actual values and the results were given in Table V.

TABLE V
OPTIMUM VALUE FOR ETHANOL PRODUCTION

Parameter	Optimum Value for Ethanol production
Substrate concentration (g/l)	33.0
pH	5.52
Temperature(^{o}C)	30.13
Ethanol concentration (g/l)	8.64

D. Leudeking-piret model

A kinetic study of batch fermentation was performed under the above optimized conditions. Maximum ethanol concentration of 8.60 g/l was obtained under optimum conditions. The experimental and the predicted values were very close which reveal the correctness and the applicability of RSM. This study showed that central composite design using response surface methodology was a suitable approach to optimize the best process conditions for achieving maximum concentration of ethanol. The kinetic data obtained from central composite design using response surface methodology experiments were used to evaluate the model parameters in the Leudeking-piret kinetic model. The model parameter values obtained were then used to simulate the model to predict the concentration of ethanol. Fig. 6 shows the experimental and predicted concentration of ethanol data for Leudeking-piret model. The kinetic parameter values of β and α were found to be 0.001 and 0.791 respectively. This model is a reasonable representation of the fermentation process for the production of ethanol.

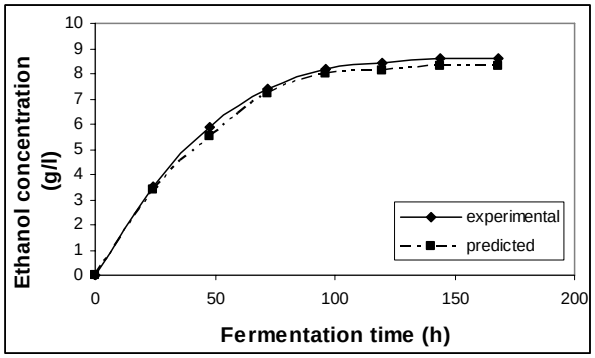

Fig. 6 Comparison between experimental results and product formation kinetic model

IV. Conclusion

The present study proves that sequential pretreated 226 white rose tapioca stem could be an effective substrate for the production of ethanol by *Fusarium oxysporum*. Statistical analysis of full factorial central composite design of the experiments revealed that the substrate concentration was the most significant variable compared to the pH and temperature. Ethanol concentration was found to decrease with an increase in substrate concentration. Studies indicated that the statistically designed experiments using RSM can be applied to similar fermentations for routine optimization. Leudeking-piret model was found to represent accurately with the experimental data of product formation kinetics.

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