KINETIC AND OPTIMIZATION STUDIES ON THE BIOCONVERSION OF LIGNOCELLULOSIC MATERIAL INTO ETHANOL

Sasikumar Ezhumalai † and Viruthagiri Thangavelu

In the present study, classical statistical tool Response Surface Methodology (RSM) was adopted for the optimization of process variables in the bioconversion of pretreated sugarcane bagasse into ethanol by cellulase and *Candida wickerhamii* MTCC 3013 based on Central Composite Design (CCD) experiments. A 2^3 five level CCD with central and axial points was used to develop a statistical model for the optimization of process variables such as incubation temperature (25 – 45°) X_1, pH (5.0 – 7.0) X_2, and fermentation time (24 – 120 h) X_3. Data obtained from RSM on ethanol production were subjected to analysis of variance (ANOVA) and analyzed using a second-order polynomial equation, and isoresponse contour plots were used to study the interactions among three relevant variables. Maximum response for ethanol production was obtained when applying the optimum values for temperature (33°C), pH (5.7), and fermentation time (104 h). Maximum ethanol concentration (4.28 g/l) was obtained from 50 g/l pretreated sugarcane bagasse at the optimized process conditions in aerobic batch fermentation. Various kinetic models such as Modified Logistic model, Modified Logistic incorporated Leudeking – Piret model, and Modified Logistic incorporated Modified Leudeking – Piret model were evaluated and the constants were predicted.

Keywords: Sugarcane bagasse; Ethanol; Optimization; Candida wickerhamii; Kinetics;

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INTRODUCTION

With industrial development growing rapidly, there is a need for environmentally sustainable energy sources. Bioethanol (ethanol from biomass) is an attractive, sustainable energy source for transporation fuel. Based on the premise that fuel bioethanol can contribute to a cleaner environment and with the implementation of environmental protection laws in many countries, demand for this fuel is increasing. Efficient ethanol production processes and cheap substrates are needed. Current ethanol production processes using crops such as sugarcane and corn are well established; however, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel (Zaldivar et al. 2001; Cardona and Sanchez 2007). One of the major lignocellulosic materials to be considered in tropical countries is sugarcane bagasse, the fibrous residue obtained after extracting the juice from sugar cane (*Saccharum officinarum*) in the sugar production process. Sugarcane bagasse is

accumulated in large quantities at cane-to-sugar processing plants and consists approximately of 50% cellulose, 25% hemicellulose, and 25% lignin. The bagasse produced is traditionally utilized for in-house energy production (Pandey et al. 2000; Haagensen and Ahring 2002).

There are major limitations to efficient ethanol production from agricultural residues; these limitations include the close physical and chemical associations between lignin and plant cell wall polysaccharides, together with cellulose crystallinity. Lignin forms a protective shield around cellulose and hemicellulose, protecting the polysaccharides from enzymatic degradation. To convert the biomass into ethanol, the cellulose must be readily available for cellulase enzymes. Thus, by removing the lignin, the cellulose becomes vulnerable to enzymes and allows the yeast to convert the glucose into ethanol during fermentation. Therefore, a pretreatment must be applied to degrade the lignin in the sugarcane residue, decrease cellulose crystallinity, and increase the surface area for enzymatic activity (Dawson and Boopathy 2007). Steam explosion was selected as the processing technology because of recent reports that steam explosion renders biomass more readily digestible by enzymes. Furthermore, steam explosion requires little or no chemical input and thus is environmentally benign relative to other technologies, such as acid hydrolysis; environmental concerns are of paramount importance (Morjanoff and Gray 1987).

Enzymatic hydrolysis is a promising approach for obtaining sugars from lignocellulosic materials. This is because it has the advantages of reduced sugar loss through side-reactions, and it is milder and more specific compared to most alternatives. But the low enzymatic accessibility of the native cellulose is a key problem for biomass-to-ethanol processes (Sun and Cheng 2002; Adsul et al. 2005). There are several technologies available for the conversion of lignocellulosics to fuel ethanol. The main difference between these technologies is the catalyst used for the break-down of polysaccharides in the raw material. Simultaneous Saccharification and Fermentation (SSF) processing is an ideal method of producing ethanol from lignocellulosic materials. In this process, a cellulose hydrolyzing enzyme (cellulase) is combined with an ethanol producing organism (yeast) to carry out simultaneous hydrolysis of cellulose to glucose and the conversion of glucose to ethanol in the same reactor (Ballesteros et al. 2004). The result is improved hydrolysis rates and yields of ethanol when compared to those involving separate hydrolysis and fermentation steps (Philiphidis et al. 1993).

The classical method of studying one variable at a time can be effective in some cases, but it is useful to consider the combined effects of all the factors involved. The Response Surface Methodology (RSM), based on statistical principles, can be employed as an interesting strategy to implement process conditions that drive to optimal ethanol production from pretreated sugarcane bagasse by performing a minimum number of experiments. Thus, RSM experimental design is an efficient approach to deal with a large number of variables. There are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation (Balusu et al. 2005; Jargalsaikhan and Saracoglu 2009). The present study investigates the potential use of sugarcane bagasse for ethanol fermentation using cellulase and yeast *Candida wickerhamii* MTCC 3013, which has the ability to ferment cellobiose directly to ethanol (Kilian et al.1983). The influence of process variables such as incubation temperature,

initial pH, and fermentation time on ethanol production from pretreated sugarcane bagasse was studied using a Central Composite experimental Design (CCD). Knowledge-based approaches such as Artificial Neural Network (ANN) were successfully applied for the purpose of simulation on the same experimental data used for RSM. Various kinetic models such as Modified Logistic model (growth kinetics), Modified Logistic incorporated Leudeking – Piret model (product formation kinetics) and Modified Logistic incorporated Modified Leudeking – Piret model (substrate utilization kinetics) were evaluated.

MATERIALS AND METHODS

Materials

A sugarcane bagasse sample was obtained from M.R.K. Sugar Mills Ltd. Sethiyathope, Tamilnadu, India. The bagasse sample was made into 100 mesh (0.15mm) fine powder by use of laboratory blender at 3000 rpm. The sample was preserved in a sealed plastic bag at 4°C to prevent any possible degradation or spoilage. Pure cellulose powder was used in reference of cellulose estimation and fermentation tests. The control and pretreated bagasse samples were analyzed for cellulose content using Anthrone reagent at 630 nm in a UV/Visible spectrophotometer ELICO BL 198 (Updegroff 1969). The estimated cellulose content of steam pretreated sample was 420 mg/g bagasse.

Micro-organism and Culture Conditions

Commercially available cellulase enzyme (ONOZUKA R–10) was obtained from HIMEDIA Laboratories, Mumbai. The activity of the enzyme was found to be 15 FPU/mL, and it was used throughout the experimentation. The cellulase activity was measured by the standard Mandel's method (Mandel et al. 1976). Yeast strain *Candida wickerhamii* MTCC *3013 was obtained from Microbial Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, INDIA. The culture was maintained on yeast extract agar medium. After three days incubation at 25°C the agar slants were stored at 4°C. The liquid medium for the growth of inoculum for yeast was yeast extract – glucose nutrient medium composed of 3g/L of yeast extract, 1g/l of sodium chloride, 10g/L of glucose, 2g/l of potassium dihydrogen phosphate, 0.2g/L of calcium chloride, and 1.7g/L of magnesium sulphate.

Inocula were grown aerobically in 250 mL Erlenmeyer flasks containing the above mentioned medium at 25°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 24 h. Active cells were centrifuged in a clinical centrifuge (1200 rpm), washed with sterile water, and were used as inoculum. Fermentations for ethanol production were conducted aerobically in an online monitored modular fermenter 2L capacity with a working volume of 1000mL medium. Samples were withdrawn periodically (12 h interval) for the analysis of cell mass, ethanol, and residual sugar concentrations.

Steam Pretreatment

Sugarcane bagasse sample was pretreated by autoclaving at 15 psi (121°C) for about 20 minutes. The steam treated samples were collected and filtered in crucibles,

followed by washed with distilled water under suction. Finally it was dried at room temperature before fermentation (Kaar et al. 1998).

Fermentation

Batch experiments were conducted as per the central composite experimental design for ethanol production in a fermenter (APPLIKON Biotech ADI 1025, Holland), with 2 L capacity, equipped with flat blade impeller, oxygen and pH electrodes, and temperature and dO_2 (dissolved oxygen) probe. The equipment also monitored temperature, agitation speed, gas purging flow rate, pumping rates, antifoam addition, dO_2, and the vessel level. All processing parameters were online monitored, with the aid of BioXpert Lite 1.00 software. The agitation speed (400±1 rpm) and dissolved oxygen, dO_2 (8±0.1 ppm) were kept constant during the experiments. Other parameters, such as temperature, pH, and fermentation time, were chosen as the most significant ones, considering the experimental design. After selecting those parameters, experiments were done in duplicate, for superior (+) and lower (-) levels of the experimental design, and in triplicate, for the central point (0). The process was conducted at the initial substrate concentration of 50g/l (pretreated sugarcane bagasse) with the addition of nutrient medium (without glucose) and 0.05 M Sodium phosphate buffer (pH 5.7) followed by sterilization for 15 min, at 15 psi (121°C). A cellulase dosage of 15 FPU/g bagasse was used for hydrolysis. For each experiment, 10mL of the inoculum was used, that is, 10%(v/v) of the initial working volume (1L). Samples were withdrawn periodically (12 h interval), centrifuged in a laboratory desktop centrifuge at 1200 rpm, and the supernatants were analyzed for total sugars and ethanol concentrations.

Cell Growth and Chemical Analysis

The sugarcane bagasse sample was analyzed for hemicellulose and Klason lignin content following the procedures described in NREL Standard Procedure (No.002). Cell mass was determined by direct optical density at 660 nm using a SYSTRONICS colorimeter (420 to 820 nm). The total reducing sugar was measured by the dinitrosalicylic acid (DNS) method using a UV/Visible spectrophotometer ELICO BL 198 at 510 nm (Miller, 1959). Ethanol was estimated using a NUCON 5765 Gas Chromatography (GC) with a Flame Ionization Detector (FID) and CHROMATOPAK (10% Carbowax 20M) column (3m length and 1/8 mm dia) using N_2 as the carrier gas at the rate of 20 µL per minute. The oven temperature was held at 80°C. The injector and detector temperature was maintained at 200°C. Ethanol concentration of the sample was obtained directly by using WINACDS software version 6.2.

Experimental Design and Statistical Analysis

In the Central Composite Design (CCD), the total number of experimental combinations was $2^K + 2K + n_0$, where K is the number of independent variables and n_0 is the number of repetitions of the experiments at the central point, which indicated that 20 experiments were required for this procedure. The CCD contains a total of 20 experiments with five level full factorial design and replications of the central points and axial points. The dependent variable selected for this study was ethanol concentration, Y (g/l). The independent variables chosen were incubation temperature (25 – 45°) X_1, pH

(5.0 – 7.0) X_2 and fermentation time (24 – 120 h) X_3. A mathematical model, describing the relationships among the process dependent variable and the independent variables in a second-order equation, was developed (Giovanni 1983). Design-based experimental data were matched according to the following second-order polynomial equation (1),

$$Y = b_0 + \sum_{i=1}^{k} b_i Xi + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j + e \quad (1)$$

where, *I* and *j* are linear and quadratic coefficients, respectively, while 'b' is a regression coefficient, *k* the number of factors studied and optimized in the experiment, and 'e' is random error. When developing the regression equation, the test factors were coded according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad i = 1, 2, 3, \ldots, k, \quad (2)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of the independent variable at the center point, and ΔX_i is the step change value.

The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by the *F*-test. The significance of each coefficient was determined using student's *t*-test. The student *t*-test was used to determine the significance of the parameters regression coefficients. The P-values (probability value) were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables. In general, larger magnitudes of *t* and smaller of P, indicate that the corresponding coefficient term is significant. The coefficients of the equation were determined by employing MINITAB software version 15. Analysis of variance (ANOVA) for the final predictive equation was done using the same software package. The response surface equation was optimized for maximum yield in the range of process variables using MATLAB software version 7.0.1. Isoresponse contour plots were obtained based on the effect of the levels of three parameters (at five different levels each) and their interactions on the yield of ethanol by keeping the other parameters at their optimal concentrations. From these contour plots, the interaction of one parameter with another parameter was studied. The optimum concentration of each parameter was identified based on the hump in the contour plots.

Artificial Neural Network (ANN) Modeling

Knowledge-based approaches such as artificial neural network have been successfully applied to modeling and control of various biological processes in recent years. ANN represents the nonlinearities better than the RSM does. ANN cannot produce a model equation similar to RSM but it works in the manner of a human brain and it estimates the response based on the training data in the investigated range. The first step in implementing a neural network modeling approach is to design the topology of the network. A number of design parameters affect performance and these parameters include the choice of activation function and training algorithm, training parameters such as learning rate and momentum, number of hidden layers, number of neurons in each

hidden layer, initial weights, and training duration. In general, feed-forward neural networks with one hidden layer containing a sufficiently large number of hidden neurons have been shown to be capable of providing accurate approximations to any continuous nonlinear function (Hornik et al. 1989; Anjum et al. 1997).

The choice of design parameters for a neural network is thus often the result of empirical rules combined with trial and error as detailed. The configuration of the two neural networks developed in this work were 3-5-1 structure: three input neurons are incubation temperature (°C), initial pH and fermentation time (h)-five neurons in one hidden layer-one output neuron and are determined after brief experimentation. To avoid the problem of overtraining, the data set comprising 20 experimental runs is split into two categories: a training set comprising 17 experimental runs is used to optimize the weights of the two neural networks and a validation set comprising 3 experimental runs is used to evaluate their predictive capability. Because empirical models like neural networks do not extrapolate data well, data for network training should be selected carefully if the best results are to be achieved. In this study the data selected for network training covered the lower and upper bounds of the one output neurons (y_1).

RESULTS AND DISCUSSION

Optimization of Process Variables in Ethanol Fermentation

The statistical technique RSM is widely used as a tool for checking the efficiency of several processes. In the present work it has been used with the purpose of obtaining information about the ethanol production process; consequently, a reduction in the operational variability and a cut down in operational costs can be expected. The experimental results (ethanol concentration, Y g/l), associated to the processing set-up of each independent variables are listed in Table 1. Five level central composite design matrix and the experimental responses of the dependent variable (ethanol concentration) are listed in Table 2. The regression equation coefficients were calculated and the data is fitted to a second-order polynomial equation. The response, Y (ethanol concentration) by *C.wickerhamii*, can be expressed in terms of the following regression equation (3):

$$Y = 3.713 - (0.2429\ X_1) - (0.1713\ X_2) + (0.5507\ X_3) - (0.3768\ X_1^2) - (0.3167\ X_2^2) - (0.3927\ X_3^2) + (0.2125\ X_1X_2) - (0.0550\ X_1X_3) + (0.0825\ X_2X_3) \quad (3)$$

Besides the linear effect of the ethanol concentration, Y g/l, the response surface method also gives an insight into the parameters' quadratic and combined effects. The analyses were done by using both Fisher's *F*- test and Student *t*-test statistical tools. The regression coefficient, *t* and P values for all the linear, quadratic, and combined effects with a 95% significance level are given in the Table 3. It shows that the regression coefficients of the linear term *X3,* and all quadratic coefficients of *X1, X2* and *X3* were significant at < 1% level ($p < 0.001$ for all), and the interaction coefficients were of less significance ($p < 0.005$).

Table 1. Coded and Actual Levels of the Independent Variables for Design of Experiment

Independent variables	Symbols	Coded levels				
		– 1.682	– 1	0	+1	+1.682
Temperature (°C)	X_1	25	30	35	40	45
pH	X_2	5	5.5	6.0	6.5	7.0
Fermentation time (h)	X_3	24	48	72	96	120

Table 2. Five-Level Factorial Central Composite Design and the Experimental Responses of Dependent Variable, Y (ethanol concentration, g/L)

Run No.	Coded levels			Real variables			Ethanol conc. (g/l)		
	x_1	x_2	x_3	[1]X_1	[2]X_2	[3]X_3	Exp	Pred (RSM)	Pred (ANN)
1	1.000	-1.000	1.000	40.0	5.5	96.0	4.62	4.47	4.65
2	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41
3	1.000	1.000	1.000	40.0	6.5	96.0	3.64	3.72	3.74
4	1.000	-1.000	-1.000	40.0	5.5	48.0	2.14	2.34	2.29
5	-1.682	0.000	0.000	26.6	6.0	72.0	3.51	3.48	3.26
6	0.000	-1.682	0.000	35.0	5.2	72.0	4.13	4.09	4.29
7	1.000	1.000	-1.000	40.0	6.5	48.0	1.85	1.83	1.89
8	0.000	0.000	1.682	35.0	6.0	112.3	4.44	4.54	4.31
9	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41
10	0.000	0.000	-1.682	35.0	6.0	31.6	2.68	2.49	2.75
11	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41
12	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41
13	0.000	1.682	0.000	35.0	6.9	72.0	3.64	3.59	3.66
14	-1.000	-1.000	-1.000	30.0	5.5	48.0	3.45	3.41	3.41
15	-1.000	-1.000	1.000	30.0	5.5	96.0	3.88	3.94	4.13
16	-1.000	1.000	-1.000	30.0	6.5	48.0	3.36	3.55	3.59
17	-1.000	1.000	1.000	30.0	6.5	96.0	4.01	3.85	4.19
18	1.682	0.000	0.000	43.4	6.0	72.0	2.52	2.47	2.50
19	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41
20	0.000	0.000	0.000	35.0	6.0	72.0	4.36	4.36	4.41

[1]X_1 (incubation temperature, °C) is calculated as: $X_1 = 35 + x_1 (5)$
[2]X_2 (initial pH) is calculated as: $X_2 = 6.0 + x_2 (0.5)$
[3]X_3 (fermentation time, h) is calculated as: $X_3 = 72 + x_3 (24)$

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*-statistics	*P*-value
Intercept	4.3621	0.05589	78.042	< 0.001
X_1	– 0.3013	0.03708	– 8.125	< 0.001
X_2	– 0.1504	0.03708	– 4.056	0.002
X_3	0.6085	0.03708	16.408	< 0.001
X_1X_1	– 0.4892	0.03610	– 13.552	< 0.001
X_2X_2	– 0.1816	0.03610	– 5.032	< 0.001
X_3X_3	– 0.2965	0.03610	– 8.214	< 0.001
X_1X_2	– 0.1638	0.04845	– 3.380	0.007
X_1X_3	0.3988	0.04845	8.230	< 0.001
X_2X_3	– 0.0587	0.04845	– 1.213	0.253
	R^2 = 0.985; Adjusted R^2 = 0.972			

Table 4. Analysis of Variance (ANOVA) for the Fitted Quadratic Polynomial Model for Ethanol Production

Sources of variation	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	*F*-value	*P*-value
Regression	12.6434	9	1.4048	74.80	< 0.001
Linear	6.6053	3	2.2017	117.23	< 0.001
Square	4.5240	3	1.5080	80.29	< 0.001
Interaction	1.5141	3	0.5047	26.87	< 0.001
Residual Error	0.1878	10	0.0187	-	-
Lack-of-Fit	0.1878	5	0.0187	-	-
Pure Error	0.0000	5	0.0000	-	-
Total	12.831	19	-	-	-

The statistical significance of the ratio between the mean square variation, due to regression, and the mean square residual error, was tested using analysis of variance (ANOVA). ANOVA is a statistical technique that subdivides the total variation of a set of data into components associated with specific sources of variation. The regression equation obtained form the ANOVA shows (Table 4) that the R^2 (coefficient of determination) was 0.951 (a value > 0.75 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 95.1% of the variation in the response. The 'adjusted R^2' is 0.907, which indicates that the model is good (for a good statistical model, the R^2 value should be in the range of 0 to 1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be). ANOVA of the regression model for ethanol yield demonstrated that the model was significant due to an *F*-value of 32.74 and a very low probability value (P model >F – 0.001).

In order to determine the optimal levels of each variable for maximum ethanol production, isoresponse contour plots were constructed by plotting the response (ethanol concentration) on the Z-axis against two independent variables, while maintaining other variables at their optimal levels, which is helpful for understanding both the main and the interaction effects of these two factors. The response surfaces can be used to predict the optimum range for different values of the test variables, and the major interactions between the test variables can be identified from the circular or elliptical nature of the contours. The circular nature of the contours signify that the interactive effects between the test variables are not significant and optimum values of the test variables can be easily obtained. Figures 1 through 3 show the isoresponse contour plots of the interactive effect of incubation temperature, initial pH, and fermentation time on ethanol production. The response values for the variables can be predicted form these plots. The effect of incubation temperature and pH on ethanol production, while the other variable (fermentation time) was fixed at its central level (72 h), is shown in Fig. 1. According to Fig. 1, the contours around the stationary point were elliptical and it became elongated more and more along the temperature axis, which meant that a small change of the response value would require a small move along the temperature axis. It was evident that the ethanol concentration steadily decreased with increasing incubation temperature upto 45°C and at low pH level. On the other hand, at high temperature, the increase in the response value was negligible as the pH value was increased. So a lower temperature and lower pH value enhance the ethanol yield. The significant interaction between incubation temperature and initial pH were apparent not only from the elliptical nature of the contour plot, but also from the low probability value (P value is 0.028; since the P value for the interaction effects were < 5% level). The other pair of the independent variables incubation temperature and fermentation time shows a less interactive effect (Fig. 2) while keeping the third independent variable, initial pH, at 6.0. From Fig. 2, it was evident that the interactive effects between the test variables were less significant not only from the circular nature of the contour plot and also from the high probability value (P - 0.520). Then the optimum values of the test variables can be easily obtained form this type of circular contour plot. Figure 3 shows a similar effect, that the variables initial pH and fermentation time show a less interactive effect in the ethanol fermentation while keeping the third variable incubation temperature constant at 35°C and found that the test variables were less significant. The results show 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 is more significantly affected by incubation temperature and initial pH than other pair of variables in the ethanol fermentation by SSF process (Harikrishna and Chowdary 2000).

The matching quality of the data obtained by the model proposed in equation (3), was evaluated by considering the correlation coefficient, R^2, between the experimental and modeled data. The mathematical adjustment of those values generated a $R^2 = 0.95$, revealing that the model would explain very well 95% of the overall effects and only 5% was not explained. In ANN modeling the R^2 value between the experimental and predicted responses is determined as 0.985, revealing that the model was not able to explain only 1.5%.

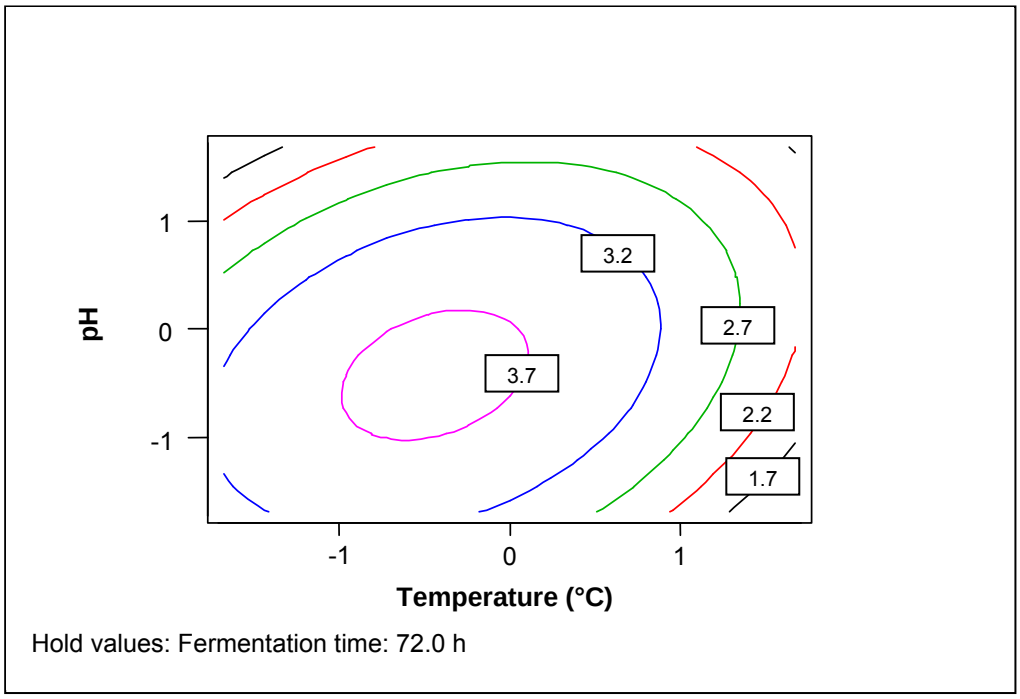

Fig. 1. Isoresponse contour plot for the effect of incubation temperature versus initial pH on ethanol production

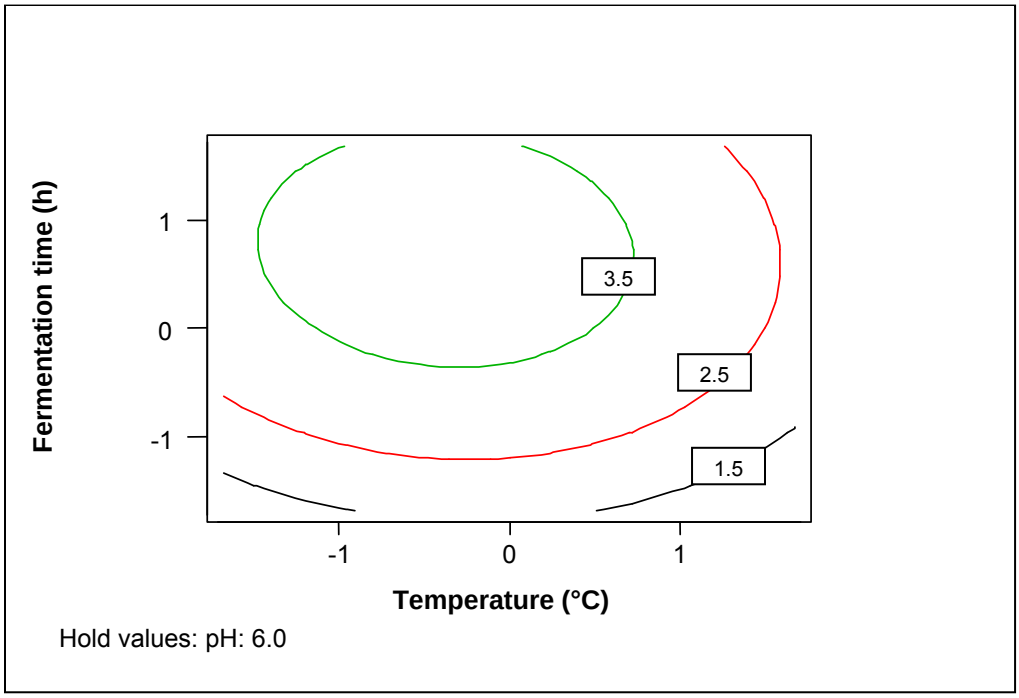

Fig. 2. Isoresponse contour plot for the effect of incubation temperature versus fermentation time on ethanol production

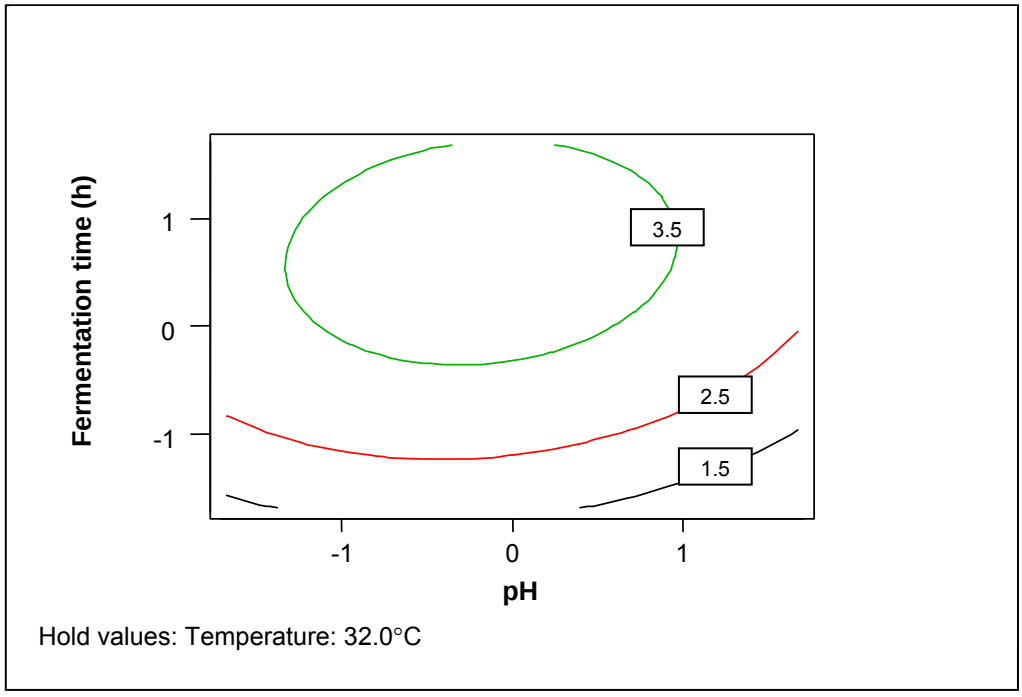

Fig. 3. Isoresponse contour plot for the effect of initial pH versus fermentation time on ethanol production

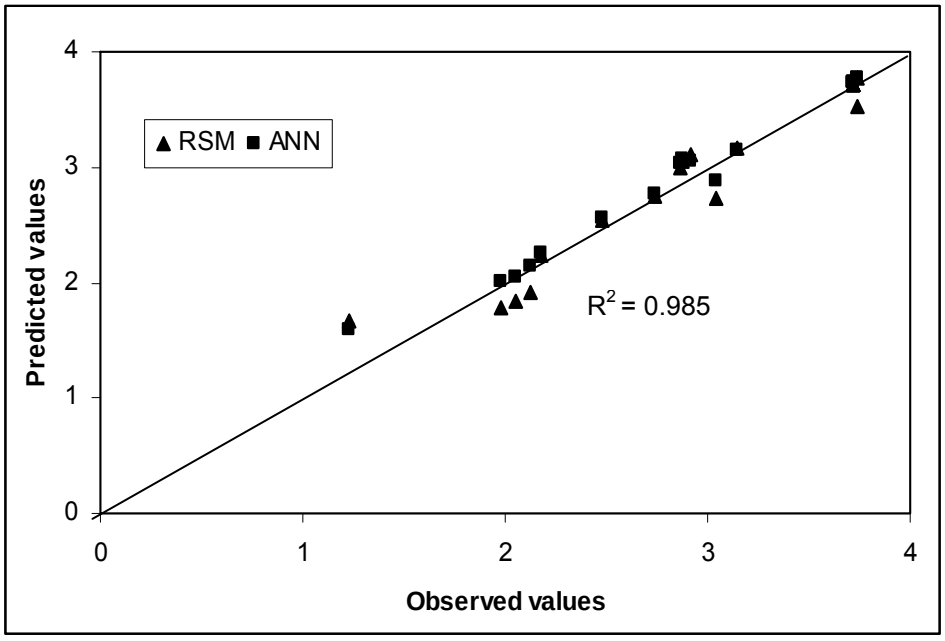

Fig. 4. Parity plot showing the distribution of experimental versus predicted values of Y (ethanol conc.) by RSM and ANN

The increase in the number of experimental points in the training the data set improved the network's performance. The parity plot shows a satisfactory correlation between the experimental and predictive values of ethanol concentration by RSM and ANN modeling (Fig. 4). From equations derived by differentiating Equation 2, the optimum values for the independent variables obtained were incubation temperature 33°C, pH 5.7, and fermentation time 104 h. Based on the model, the optimal working conditions were obtained to attain high ethanol yield. Response analysis revealed the maximum ethanol concentration (4.28 g/l) by *C.wickerhamii* could be achieved at the optimum process conditions at 104 h. The final ethanol yield was compared with the reported results and it was found that an increase in ethanol yield by about 40% was achieved (Harikrishna and Chowdary, 2000; Harikrishna et al. 2001).

KINETICS AND MODELING

Kinetic studies are necessary to gain a basic understanding of any fermentation, and they are very useful for efficient economical production of metabolites (Dhanasekar et al.2003; Sasikumar and Viruthagiri 2008). The validity of the proposed model under different experimental conditions has been tested. The cell mass, product formation, and substrate utilization kinetics using *C.wickerhamii* with different parameters were studied.

Modified Logistic Model (growth)

Under optimal growth conditions and when the inhibitory effects of substrates and product play no role, the rate of cell growth is given by equation (4)

$$\frac{dX}{dt} = \mu_0 X \quad (4)$$

where μ_o is a constant defined as the initial specific growth rate.

Table 5. Model Parameters for Ethanol Production

Model parameters	Models		
	Modified Logistic	Modified Logistic incorporated Leudeking – Piret	Modified Logistic incorporated Modifed Leudeking – Piret
[4]μ0	0.21	-	-
[5]r	0.550	0.580	0.720
[6]α	-	0.556	0.557
[7]β	-	0.009	0.030
[8]γ	-	6.996	8.864
[9]η	-	0.055	0.019
Avg. error	7.58 %	4.69 %	4.79 %
R^2	0.989	0.995	0.992

[4]Initial specific growth (h^{-1})
[5]Inhibitory effect index
[6]Non-growth associated constant for substrate
[7]Substrate consumption (g substrate / g biomass h)
[8]Growth associated constant (g product / g biomass)
[9]Non-growth associated constant (g product / g biomass h)

The logistic model equation implies that the growth rate increases with increase in cell mass concentration and is independent of the substrate concentration. A modified form of logistic equation is used to describe the cell growth kinetics by introducing an index of the inhibitory effect 'r', which accounts for the deviation of growth from the exponential relationship metabolites (Dhanasekar et al. 2003; Sasikumar and Viruthagiri 2008), as equation (5)

$$\frac{dX}{dt} = k\left[1 - \left(\frac{X}{X_{\max}}\right)^{r}\right]X \quad \text{for r>0} \tag{5}$$

When r = 0, there will be a complete inhibition of cell growth; when r = 1, equation (5) reduces to a logistic model equation; when r ranges between 0 and 1, equation (5) describes a higher degree of inhibition compared to logistic growth; when r >1, the growth lies between exponential and logistic patterns. Equation (5) was rearranged and integrated by using partial fraction method with the initial conditions, $X = X_0(t = 0)$, which gives equation (6)

$$X_t = \frac{X_m^r e^{\mu_0 rt}}{1 - \frac{X_0^r}{X_m^r(1 - e^{\mu_0 rt})}} \tag{6}$$

The model parameter values were evaluated using MATLAB software version 7.0.1 program and are shown in Table 5. A better prediction of cell mass concentrations was obtained using the modified logistic model and it was most suited for ethanol production with the minimum average error of 4.56 %.

Modified Logistic Incorporated Leudeking – Piret Model (product formation)

A Modified Logistic incorporated Leudeking – Piret model was developed by rearranging and integrating the Leudeking – Piret model with two initial conditions, $X=X_0$ (t=0) and $P = P_0$ (t=0), giving equation (7)

$$P_t = P_0 + \alpha\left\{\left[\frac{X_0^r e^{\mu_0 rt}}{1 - \frac{X_0^r}{X_m^r}\left(1 - e^{\mu_0 rt}\right)}\right]^{1/r} - X_0^r\right\} + \frac{\beta X_m^r}{\mu_0}\ln\left[1 - \frac{X_0^r}{X_m^r}\left(1 - e^{\mu_0 rt}\right)\right] \tag{7}$$

The model parameter values were evaluated using the MATLAB program and are presented in Table 5. The simulation result of the Modified Logistic incorporated Leudeking – Piret model is in good agreement with the experimental data obtained from the pretreated sugarcane bagasse and the minimum average error of 5.69 %.

Modified Logistic incorporated Modified Leudeking – Piret model (substrate utilization)

The substrate utilization kinetics is the modified form of the Leudeking – Piret model which can be used for substrate utilization kinetics. Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The Modified Logistic incorporated Modified Leudeking – Piret model was developed by rearranging and integrating the Modified Leudeking – Piret model with two initial conditions, $X=X_0$ (t=0) and $S= S_0$ (t=0) gives equation (8)

$$S_t = S_0 - \gamma\left\{\left(\frac{X_0^r e^{\mu_0 rt}}{1-\frac{X_0}{X_m^r\left(1-e^{\mu_0 rt}\right)}}\right)^{1/r} - X_0^r\right\} - \frac{\eta X_m^r}{\mu_0}\ln\left[1-\frac{X_0}{X_m^r}\left(1-e^{\mu_0 rt}\right)\right] \quad (8)$$

The model parameter values shown in Table 5 are then used to simulate the experimental data of substrate concentration at any time during the entire course of fermentation. Better substrate utilization kinetics is obtained using the Modified Logistic incorporated Modified Leudeking – Piret model (Eqn. 8) and is well suited for ethanol production from pretreated sugarcane bagasse with a minimum average error of 6.82 %.

CONCLUSIONS

Based on the present study, it is evident that the use of statistical optimization tools, response surface methodology (RSM), has helped to locate the optimum levels of the most significant parameters for ethanol production, with minimum effort and time. Maximum ethanol concentration (4.28 g/l) was obtained from 50 g/L of pretreated sugarcane bagasse at the optimized conditions (incubation temperature 33°C, initial pH 5.7 and fermentation time 104 h) by using yeast strain *C. wickerhamii.* Modified logistic model, Modified Logistic incorporated Leudeking – Piret model, and Modified Logistic incorporated Modified Leudeking – Piret model were attempted for representing the batch growth kinetics, product formation kinetics and substrate utilization kinetics respectively. The results of the process simulation from the various models using the experimental data were compared and found to predict more accurately during the entire course of fermentation.

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