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RESEARCH PAPER

Optimization of fermentation parameters using response surface methodology for ethanol production from pretreated cumbu napier grass

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ABSTRACT

The optimization of fermentation parameters for ethanol production by elite thermo tolerant yeast *Kluveromyces marxianus* was investigated in simultaneous saccharification and fermentation process using pretreated cumbu napier grass. The physical chemical characterization of the substrate expressed that the cellulose content was about 48.7 per cent and the hemicelluloses content was 20 per cent. The fermentation parameters such as commercial cellulase concentration, pH, temperature and fermentation time using the RSM were optimized for enhancing ethanol yield using central composite design. The optimal level of each parameter for maximum ethanol yield by the thermo tolerant yeast was determined. From the analysis conducted by Design Expert software version 8.0.7.1, the optimum combinations were commercial cellulase enzyme concentration, pH, temperature and fermentation time of 20 FPU g^{-1} substrate, 5, 42.5°C and 108 h. Under optimum conditions, the maximum conversion efficiency predicted by the model was 32.6 g l^{-1} of ethanol. The model computed for R^2 value was 0.9443 per cent indicating that it was appropriate and could be useful to predict the levels of varaibles to achieve maximum ethanol yield. Validation of the predicted results were done and the experimental values correlated well with that of predicted results.

Key Words : Bioethanol, Optimization, Response surface method, Central composite design

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In the recent times, there is an increasing demand for the alternative renewable sources of fuels due to excessive consumption and depletion of fossil fuels. Currently, ethanol is mainly produced from starch materials which are not enough to meet the demand of fuel ethanol.

Lignocellulosic materials are cheap renewable resources which is available in large quantities globally. The variety of materials such as agricultural residues, fruit and vegetable wastes, wood, municipal solid waste, etc., to meet out the global demand of ethanol. So bioethanol from lignocellulosic substrates could be a key

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alternative and sustainable one.

Cumbu napier hybrid fodder grass is one of the herbaceous lignocellulose having the capacity to produce bioethanol. It is a perennial crop with the characteristic of profuse tillering, high yield potential of 400 tonnes per ha and quick regeneration capacity. It contains 6 per cent sugar and 74-78 per cent holocellulose (cellulose and hemicellulose) and low lignin content, which can be efficiently converted into ethanol. Bioethanol production from napier grass has been extensively studied now-a-days due to its low lignin content (Anderson *et al*., 2008; Kai *et al*., 2010; Zhang *et al.,* 2011 and Yasuda *et al*., 2012).

Overall the steps involved in fuel ethanol production from lignocellulosic biomass consists of feedstock preparation, pretreatment, fractionation, enzymatic hydrolysis (saccharification), fermentation, product recovery (Saha, 2004). Conversion of lignocellulosic sugar hydrolysate into ethanol requires many variables apart from fermentable sugars which in right balance give optimum product yield. Statistical screening provides a proper assessment of key process variables to improve product yield. Response surface methodology explores the relationships between several operating variables and one or more response variables and has been widely applied for optimization of ethanol production from various substrates (Uncu and Cekmcelioglu, 2011).

Hence, the present study was carried out to optimize and validate the various factors by Response Surface Method (RSM) employing Central Composite Design (CCD) for improved ethanol production from acid pretreated cumbu napier grass. Commercial cellulase enzyme concentration, pH, temperature and fermentation time are the important parameters considered for bioethanol production.

RESEARCH METHODOLOGY

Materials :

The Cumbu Napier fodder grass was obtained from the Department of Forage Crops, Tamil Nadu Agricultural University. The moisture content was reduced drastically by introducing the substrates to the interior of the Tunnel drier until it reaches the brittle texture. After attaining a brittle texture, the substrate was cut into about 10 cm length and pulverized by using the Willey mill (M/s. Khera, India). After accomplishing a disintegrated biomass, the substrate was sieved to different micron sizes using sieve shaker (M/s. Jayanth, India). The physio-chemical characteristics of the substrate such as cellulose, hemicellulose, lignin, reducing sugars, moisture and ash content were analysed using the standard protocol.

Pretreatment of the substrate :

Five grams of the sieved < 250 µ substrate was taken in a 250 ml conical flasks and 100 ml of 3 per cent of concentrated H_2SO_4 was added to the flask and incubate for 3 hours to hydrolyze the substrate and the flask was kept in autoclave at 121°C for 30 min followed by sudden depressurization by fully opening the steam exhaust valve of autoclave. The flasks were cooled to the room temperature (28°C) and the hydrolyzate was filtered through the Whatman No.1 filter paper.

Organism :

The organism used in the study is elite thermo tolerant yeast TY16 *Kluyveromyces marxianus* isolated from spent wash storage site in Sakthi distilleries, Erode. The stock culture was maintained in YPD agar medium.

Experimental design :

RSM using Central Composite Design (CCD) for four factors with replicates at the centre point and star points were used in the investigation for optimizing SSF process parameters. The variables used were commercial cellulase enzyme concentration, E (FPU g^{-1} substrate), pH P, temperature T (°C) and fermentation time H (h) each at five coded levels. The actual levels of variables were selected based on the initial levels as the centre points.

The natural, coded levels and interval of variation of the independent variables in the experimental plan for the optimization of fermentation process was given in the Table 1.

A total of 27 experimental trials that included 16 trials for factorial design, 8 trials for axial points and 3 trials for replication of the central points were performed (Table 2). The response value, ethanol yield is the average of triplicates. The response value was ethanol yield obtained from 27 experimental runs and the best treatment was selected for SSF process. The maximum and minimum variable levels were selected on the basis of preliminary studies. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors.

The experiments were conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

Statistical modeling of responses :

Empirical statistical modeling was used to develop an appropriate approximating model between the response (y) and independent variables (X_1, X_2, X_3, ……X_k).

In general the relationship is

$$Y = f(X_1, X_2, X_3, \ldots\ldots X_k) + £ \quad \ldots(1)$$

The variables X_1, X_2, X_3, ……X_k in the Eqn. 1 are usually called the natural variables in RSM. It is convenient to transform the natural variables to coded variables x_1, x_2, x_3, ……$x_{k,,}$ which are dimensionless having mean zero and variance equal to 1. In terms of the coded variables, the response function (Eqn. 1) will be written as

$$y = f(x_1, x_2, x_3, \ldots\ldots x_k) + £ \quad \ldots(2)$$

The function 'f' is called the response surface. The form of the function 'f' is unknown. The term 'ε' represents other sources of variability not accounted for in 'f' and usually it is treated as statistical error. As the form of the response function 'f' is unknown, it must be approximated. Polynomials are often chosen because they usually offer an adequate approximation of the true response surface. In many cases, either a first order or a second order model as shown below is used.

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \varepsilon \quad \ldots(3)$$

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j + \varepsilon \quad \ldots(4)$$

The first model (Eqn. 3) is likely to be appropriate when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variables space in a location where there is no curvature in 'f'. If there is a curvature in the system, then a polynomial of higher degree such as second order model (Eqn. 4) must be used.

To find out the effect of the independent variables on the dependent variables, the first order linear equation (Eqn. 4) was fitted between 'x' and 'y'. For optimization of the independent variables and to check the sufficiency of the experimental design, the second order non-linear regression equation (Eqn. 5) was fitted between dependent and independent variables.

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_{3 + \ldots\ldots} b_k x_k \quad (5)$$

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \quad (6)$$

where,

y is the response variable

b_0, b_1, b_2 and b_3 are regression co-efficients of linear terms

b_{11}, b_{22}, and b_{33} are regression co-efficients of quadratic terms

b_{12}, b_{13} and b_{23} are regression co-efficients of cross-product terms

x_1, x_2, and x_3 are the coded values of the independent variables X, *viz.*, pH (X_1), temperature (X_2), and incubation time (X_3), respectively. The quality of fit of the second order equation was expressed by the co-efficient of determination R^2, and its statistical significance was determined by F-test. The significance of the regression co-efficient was determined by p-value. The co-efficients of the equation were determined by employing Design Expert software Version 8.0.7.1. Analysis of variance (ANOVA) for the final predictive equation was done using Design Expert software. The response surface analysis was made keeping one independent variable at middle level while changing the other two. The response surface equation was used to optimize the independent variables for the response variables such as ethanol yield.

RESULTS AND REMONSTRATION

The physio-chemical properties of the cumbu napier was analyzed and found to contain 48.7 per cent of cellulose, 20 per cent Hemicellulose, 16 per cent lignin and 5 per cent ash, respectively. The holocellulose content was about 68.7 per cent which showed that this substrate has more efficiency to produce more amount of ethanol.

The optimization of SSF process for ethanol production from cumbu napier was carried out by employing 5 per cent substrate concentration and elite ethanologenic thermotolerant yeast TY16 *Kluveromyces marxianus* with different levels of commercial cellulase concentration, pH, temperature and fermentation time using the RSM. The central composite design matrix and the experimental responses of the dependent variable (ethanol production) are listed in Table 2. The data

obtained were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms. Data obtained from the experiments (Table 2) were analyzed by multiple regression using Design Expert software version 8.0.7.1. The centre point in the design was repeated for estimation of errors. The following equation was obtained:

Final equation in terms of coded factors

$$Y = 31.97 + 1.22 A + 0.46 B - 4.03 C - 0.071 D + 0.031 AB + 0.17 AC - 0.13 AD + 0.44 BC + 0.64 BD + 0.081 CD - 2.05 A^2 - 2.24 B^2 - 4.34 C^2 - 1.57 D^2 \quad .. (7)$$

where,

Y is the ethanol production,

A is concentration of commercial cellulase enzyme,

B is pH value,

C is Temperature and

D is Fermentation time

Based on the experimental response, the ethanol production varies from 6.30 g l^{-1} to 32.6 g l^{-1}, standard order 22 and 25 had the minimum and maximum level of

Table 1 : Natural levels, codes and intervals of variation of the independent variables in the design of experiments

Factors	Codes	Levels					Interval of variation
		-1	-α	0	+α	+1	
Cellulase enzyme concentration (FPU g^{-1} substrate)	*E*	15.00	10.00	20.00	30.00	25.00	10
pH	*P*	4.5	4.0	5.0	6.0	5.5	1.0
Temperature (°C)	*T*	38.75	35	42.5	50	46.25	7.5
Fermentation time (h)	*H*	90	72	108	144	126	36

Table 2 : Central composite design matrix of different parameters of independent variables and their corresponding experimental and predicted values of ethanol production from cumbu napier

Standard order	Independent variables				Ethanol production (g l^{-1})	
	Cellulase enzyme concentration (FPU)	pH	Temperature (°C)	Fermen-tation time (h)	Observed	Predicted
1	15.00	4.50	38.75	90.00	25.20	25.43
2	25.00	4.50	38.75	90.00	26.40	27.73
3	15.00	5.50	38.75	90.00	24.30	24.11
4	25.00	5.50	38.75	90.00	25.50	26.54
5	15.00	4.50	46.25	90.00	16.50	15.98
6	25.00	5.50	46.25	90.00	18.60	18.96
7	15.00	5.50	46.25	90.00	17.20	16.44
8	25.00	5.50	46.25	90.00	19.30	19.55
9	15.00	4.50	38.75	126.00	22.80	24.10
10	25.00	4.50	38.75	126.00	24.10	25.88
11	15.00	5.50	38.75	126.00	24.70	25.36
12	25.00	5.50	38.75	126.00	25.20	27.26
13	15.00	4.50	46.25	126.00	15.00	14.98
14	25.00	4.50	46.25	126.00	15.70	17.43
15	15.00	5.50	46.25	126.00	17.80	18.01
16	25.00	5.50	46.25	126.00	19.80	20.59
17	10.00	5.00	42.50	108.00	20.50	21.33
18	30.00	5.00	42.50	108.00	29.60	26.21
19	20.00	4.00	42.50	108.00	23.90	22.10
20	20.00	6.00	42.50	108.00	24.70	23.95
21	20.00	5.00	35.00	108.00	25.50	22.68
22	20.00	5.00	50.00	108.00	6.30	6.56
23	20.00	5.00	42.50	72.00	25.40	25.81
24	20.00	5.00	42.50	144.00	28.50	25.53
25	20.00	5.00	42.50	108.00	32.60	31.97
26	20.00	5.00	42.50	108.00	31.40	31.97
27	20.00	5.00	42.50	108.00	31.90	31.97

ethanol production respectively. The ANOVA results of quadratic regression model for ethanol production are described in Table 3. The analysis of variance of the quadratic regression model demonstrated that the equation was a highly significant model, as was evident from the Fisher's F test with a very low probability value [(P model > F) = 0.0001]. The model F-value of 14.52 implied that the model was significant. The fitness of the model was examined by the co-efficient of determination R^2 (0.9443), which implied that the sample variation of more than 94 per cent was attributed to the variables and only 6 per cent of the total variance could not be explained by the model. The closer the R^2 value is 1, the better the model is fit to experimental data, the less is the distance between the predicted and the observed values. The value of the adjusted determination co-efficient (Adj R^2 = 0.8793] was also very high in supporting the high significance of the model. A lower value of co-efficient of variation (CV= 8.99%) showed the experiments conducted were precise and reliable. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 16.551 indicated an adequate signal, which implied that this model could be used to navigate the design space. The significance of each co-efficient, which was measured by t test and P value are shown in Table 4. The larger the magnitude of t test and smaller the P values are, the corresponding co-efficients are more significant. Values of "Prob > F" less than 0.0500 indicated model terms were significant whereas values greater than 0.1000 indicated the model terms were not significant. In this case, concentration of commercial cellulase enzyme and temperature had significant effects on ethanol production whereas the effect of pH value and fermentation time was not significant. There were no significant interactions between any two factors. The quadric effects of commercial cellulase concentration, pH, temperature and fermentation time were significant.

Table 3 : Analysis of variance (ANOVA) for optimization of different parameters for maximum ethanol production from cumbu napier

Sources	Sum of squares	Degree of freedom	Mean square	F value	Significance F
Regression	862.28	14	61.59	14.52	< 0.0001
Residual	50.89	12	4.24	-	-
Total model	913.17	26	-	-	-

R^2 = 0.9443

Table 4 : Regression co-efficient for optimization of different parameters for maximum ethanol production from cumbu napier

Factors	Regression co-efficient	Standard error	P value
Intercept	31.97	1.19	< 0.0001
A-Enzyme conc.	1.22	0.42	0.0132
B-pH	0.46	0.42	0.2928
C-Temp	-4.03	0.42	< 0.0001
D-Time	-0.07	0.42	0.8690
AB	0.03	0.51	0.9526
AC	0.17	0.51	0.7487
AD	-0.13	0.51	0.8031
BC	0.44	0.51	0.4056
BD	0.64	0.51	0.2350
CD	0.08	0.51	0.8772
A^2	-2.05	0.45	0.0006
B^2	-2.24	0.45	0.0003
C^2	-4.34	0.45	< 0.0001
D^2	-1.57	0.45	0.0041

Table 5 : Experiment employing different parameters for maximum ethanol production from cumbu napier as predicted by model

Dependent variable	Independent variables				Ethanol production (g l^{-1})	
	Cellulase enzyme concentration (FPU)	pH	Temperature (°C)	Fermentation time (h)	Observed	Predicted
	20	5	42.5	108	32.6	31.97

Graphical representation of 3D response surfaces are shown in Fig. 1 to highlight the roles played by various factors commercial cellulase enzyme concentration, pH, temperature and fermentation time on ethanol production. Accordingly, three-dimensional graphs were generated for the pair-wise combination of the 3 factors, while keeping the other factors at their center point level. The 3D surface response for independent variables commercial cellulase enzyme concentration, pH, temperature and fermentation time on ethanol production suggested that ethanol production was affected by all the variables. The factors *viz.*, commercial cellulase enzyme concentration and pH showed the positive effect on ethanol production, whereas temperature and fermentation time portraited the negative effect. Ethanol production increases with increase in commercial cellulase enzyme concentration and pH, however, decreases with temperature and fermentation time. From the analysis conducted by Design Expert software version 8.0.7.1, the optimum combinations were commercial cellulase enzyme concentration, pH, temperature and fermentation time of 20 FPU g^{-1} substrate, 5, 42.5°C and 108 h. Under optimum conditions, the maximum conversion efficiency predicted by the model was 32.6 g l^{-1} of ethanol (Table 5).

Operation parameter optimization by the traditional one factor at a time requires a considerable amount of work and time. An alternate strategy is a statistical approach such as RSM, involving minimum number experiments for a large number of factors. RSM has been shown to optimize the process in many works (Zheng *et al.,* 2008). In this study, the optimization of SSF process for ethanol production from selected lignocellulosic substrates were carried out by employing 5 per cent substrate concentration and elite ethanologenic thermo tolerant yeast TY16 with different levels of commercial cellulase concentration, pH, temperature and fermentation time using RSM.

Temperature was the crucial factor in SSF, because of the differences in optimum temperature of saccharification (50°C) and that of yeast fermentation (35°C). The rate of saccharification was slow at 35°C but the ethanol yield was higher. Although the maximum saccharification was obtained at 45°C, the rate of ethanol production was very low and a significant proportion of sugars remained unmetabolized. Temperatures above 45°C adversely affected ethanol

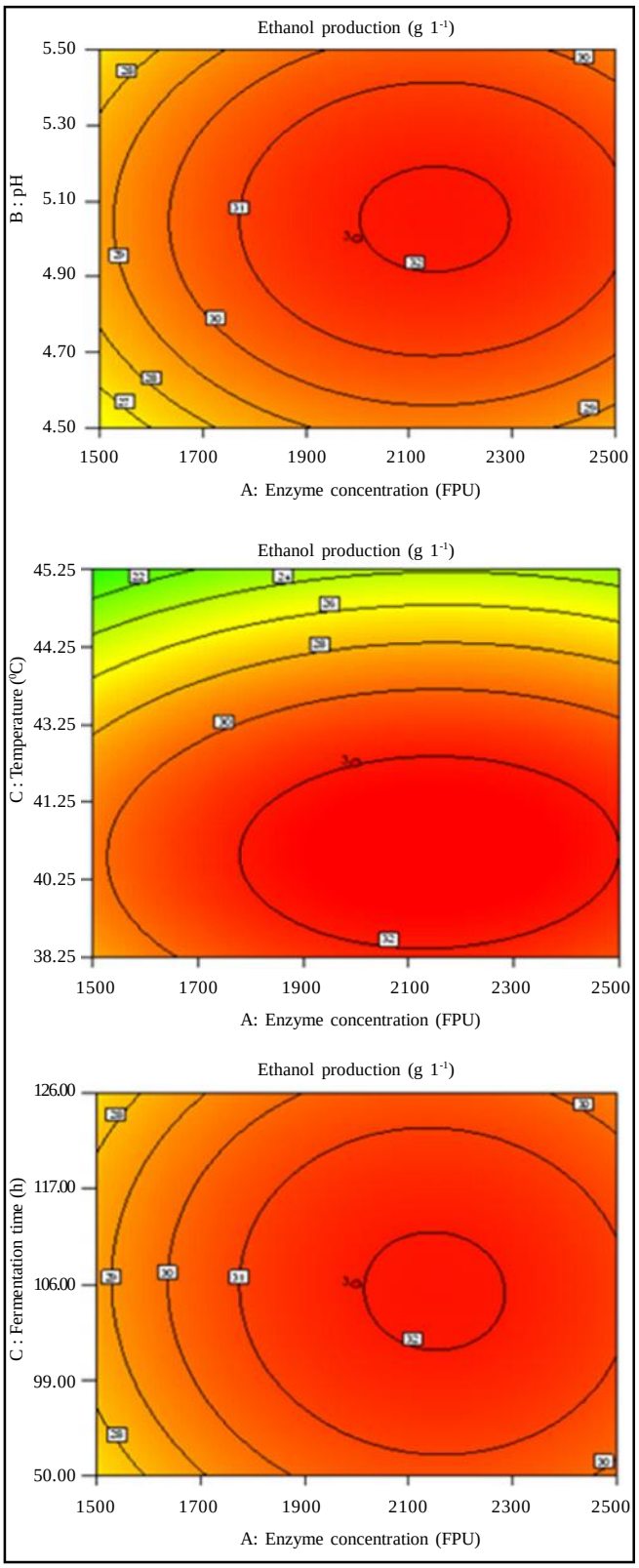

Fig. 1 : Response surface curve showing the effect of different parameters on ethanol production from cumbu napier grass

production because the yeast cells not able to work at that high temperature Hence, 42.5°C was chosen as the optimum temperature for maximum ethanol production. Previously Harikrishna *et al*. (1998) reported the similar results.

In the present study, ethanol concentration did not increase significantly at cellulase concentration greater that 20 FPU g^{-1} substrate. Reduction of cellulase enzyme loading lesser than the concentration of 20 FPU g^{-1} substrate in SSF resulted in reduced glucan hydrolysis and ethanol production. Suryawati *et al*. (2008) carried out SSF at pH 5.5 and pH 4.8 and compared in terms of ethanol yield. In SSF with pH 5.5, final ethanol yield of 92 per cent was obtained whereas pH 4.8 resulted in lower ethanol yield of 79 per cent. This showed that pH had an effect on ethanol production. Similarly, in the present study also, the ethanol yield was higher in the range of pH 5.

Sasikumar and Viruthagiri (2008) also studied on optimization of process conditions using RSM for ethanol production from pretreated sugarcane bagasse. They selected substrate concentration, pH, incubation temperature and fermentation time as variables. They obtained maximum ethanol concentration of 32.6 g l^{-1} from pretreated sugarcane bagasse under optimized conditions of 35°C, pH 5.5 in 72 h. The result varies from the present study due to the nature and composition of substrate and also the activity of enzyme and yeast used.

Mohan *et al*. (2012) also employed central composite design to optimize the fermentation medium conditions for ethanol yield in SSF process using pretreated sugarcane bagasse and indicated that the model was appropriate and could be useful to predict the level of variables to achieve the maximum ethanol yield.

Graphical representation of effect of different variables on ethanol production showed the cellulase enzyme concentration increased from 10 to 30 FPU g^{-1} substrate, the hydrolysis rate increased and attained maximum hydrolysis at 20 FPU g^{-1} substrate in cumbu napier. When the temperature increased from 35°C to 50°C, ethanol concentration increased upto 45°C and the maximum ethanol concentration reached at 42.5°C. In the different ranges of pH analyzed, the pH 5 was found to be the best for maximum ethanol production. Among the different fermentation time, ethanol production was higher when incubated for 108 h.

Conclusion :

Optimization of cultural conditions for fermentation is a most important concern to develop a suitable process for ethanol yield. The current study using RSM on central composite design was found be an efficient model to optimize the parameters for maximum ethanol production from cumbu napier grass and substrate also proven to be the best for ethanol production.

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