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Research Article

Optimization of process variables using response surface methodology (RSM) for ethanol production from cashew apple juice by *Saccharomyces cerevisiae*

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Abstract

This study was conducted in Annamalai University, Faculty of Engineering and Technology, Department of Technology, India during 2009 to optimize the process variables in the production of ethanol from cashew apple juice using *Saccharomyces cerevisiae*. Bioethanol is an important renewable energy resource; it is an attractive, sustainable energy source to fuel additives (has higher octane number and higher heat of vaporization). The potential use of cashew apple juice in ethanol fermentation was evaluated by using the Response Surface Methodology (RSM) in this study. A 2^4 five level Central Composite Design (CCD) was used to develop a statistical model for the optimization of process variables such as substrate composition (50 – 90% v/v) X_1, pH (5.0 – 7.0) X_2, incubation temperature (30 – 38°C) X_3 and fermentation time (36 – 60 h) X_4 by *Saccharomyces cerevisiae* MTCC 170. The design contains a total of 31 experimental trials with a full factorial design fashion and the replications of the central points. Data obtained from RSM on ethanol production were subjected to the Analysis of Variance (ANOVA) and analyzed using a second order polynomial equation resulted in the optimized process parameters of 62% (v/v) as substrate concentration, pH – 6.5, temperature 32°C and fermentation time of 42h. Maximum ethanol concentration (15.64 g/l) was obtained at the optimized conditions in aerobic batch fermentation.

Keywords: ethanol; Central Composite Design (CCD); Response Surface Methodology (RSM); Cashew apple juice

Introduction

One of the greatest challenges for society in the 21 century is to meet the growing demand for energy for transportation, heating, and industrial processes and to provide raw material for the industry in a sustainable way. An increasing concern for the security of oil supply has been evidenced by increasing oil prices, which, during 2006 approached US$80 per barrel [1]. More importantly, the future energy supply must be fulfilled with a simultaneous substantial reduction of green house gas emissions [2]. Ethanol satisfies that requirement because its production and combustion do not contribute significantly to the total amount of carbon dioxide in the atmosphere [3]. Non-renewable energy sources, i.e. fossil fuels represent the most exploited forms of energy today and it has been calculated that at the present rate of production fossil fuels would be exhausted in the next century. In recent years, fermentative production of ethanol from renewable resources has received attention due to increasing petroleum shortage. Hence there is a need to develop and implement viable technologies for the production of alternative renewable energy and feedstock. Today, however, many of the technologies for the production of alternative fuels such as bioethanol are not competitive with the cheap fossils fuels available. Despite this, some commercial interest and research continues because of the abundance of raw materials and the prediction that the energy economics will change near future to favor biofuels [4, 5].

Cashew is produced in around 32 countries of the world, and the major cashew apple producing countries are Vietnam – 8.4 million tons, Nigeria – 5 million tons, India – 4 million tons, Brazil – 1.6 million tons and Indonesia – 1 million tons (based on FAO 2004). India now accounts for about 40 percent of world cashew production. Considering that the use of agro-industrial residues can contribute for the reduction of production costs, cashew apple (false fruit, *Anacardium occidentale* L.) appears as an alternative raw material for ethanol production, due to its vast availability and high concentration of reducing sugars (30% of fructose and glucose), which can be utilized for fermentation of ethanol [6, 7]. It can also be squeezed for fresh juice, which can then be fermented into cashew wine, which is a very popular drink in West Africa. In parts of India, it is used to distil cashew liquor referred to as *feni* (alcoholic drink) [8]. Cashew apple juice normally has sufficient organic nutrients and minerals (Vitamin C, calcium, iron, phosphorus, sodium and potassium) that make it suitable for ethanol production by fermentation with microorganisms (because of high mineral content of the juice, no mineral addition was necessary) [9]. Cashew apple has no commercial use value, except for its use by rural inhabitants in the production of homemade alcoholic beverages [10].

Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess. Statistical methodologies involve use of mathematical models for designing fermentation processes and analyzing the process results [11]. RSM is a powerful mathematical model with a collection of statistical techniques where in, interactions between multiple process variables can be identified with fewer experimental trials. It is widely used to examine and optimize the operational variables for experiment designing, model developing and factors and conditions optimization [12, 13]. There are various advantages in using statistical methodologies in terms of rapid and reliable short listing of process conditions, understanding interactions among them and tremendous reduction in total number of experiments. 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 alcohol production from waste cashew apple juice by performing a minimum number of experiments. In the present work, optimization of process conditions (substrate concentration, temperature, pH and fermentation time) using RSM for the production of ethanol from waste cashew apple juice by *Saccharomyces cerevisiae* MTCC 170 have been carried out and the influence of process variables on ethanol production was well studied using CCD experiments.

Materials and Methods

Materials

Waste cashew apples were brought from a factory processing cashew apples to extract the juice. Cashew apples are cut into slices in order to ensure a rapid rate of juice extraction when they are crushed in the juice press. The fruit juice is pasteurized in stainless steel pans at a temperature of 85^{o}C in order to eliminate any wild yeast. This juice, which contains high levels of tannins, was clarified by adding gelatin to remove tannins and suspended solids [14]. Then the clarified juice was filtered and treated with either sodium or potassium meta-bisulphate, to destroy or inhibit the growth of undesirable types of microorganisms such as acetic acid bacteria, wild yeast and moulds. Juice sample was filled in jars (capacity 2.5 liters) and was preserved at 4°C to prevent any possible degradation or spoilage during storage. This treated juice sample (contains 28.5% of total reducing sugars) was used throughout the experimentation

Microorganisms and culture conditions

Yeast strain *Saccharomyces cerevisiae* MTCC 170 was obtained from Institute of Microbial Technology (IMTECH), Microbial Type Culture Collection centre (MTCC), Chandigarh, India. Culture was maintained on potato dextrose-agar medium. After three days incubation at 30°C the agar slants were stored at 4°C.The liquid medium for the growth of inoculum for yeast strain was composed of 15-20 g/l glucose, fructose or sucrose, 10 g/l yeast extract, 2 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 1 g/l $MgSO_4.7H_20$.

Inocula were grown aerobically in 250 ml Erlenmeyer flasks containing the above mentioned medium at 30°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 24 h. Active cells were centrifuged in a clinical centrifuge 11806x*g* (8000 rpm) for 10 min, washed with sterile water, and were used as inoculum. Fermentations for ethanol production were conducted aerobically in an online monitored modular fermenter 3-liter capacity with a working volume of 1000ml medium. Samples were withdrawn periodically (12 h interval) for the analysis of ethanol and residual sugar concentrations.

Experimental design and statistical analysis

An uniform-precision 2^4 ($k = 4$) factorial central composite experimental design with eight star points ($F = 8$), six axial points and six replicates at the center point ($n_0 = 6$), resulting in a total of 31 experiments ($\alpha = 2$) which covers the entire range of spectrum of combinations of variables were used to optimize the chosen key variables for the ethanol production from waste cashew apple juice in an aerobic batch bioreactor. The experiments were conducted in a randomized fashion. The dependent variable selected for this study was ethanol (g/l) yield. The independent variables chosen were substrate composition (50 – 90% v/v) X_1, initial pH (5.0 – 7.0) X_2, incubation temperature (30 – 38°C) X_3 and fermentation time (36 – 60h) X_4 (Table 1).

A mathematical model, describing the relationships among the process dependent variable and the independent variables in a second-order equation, was developed [15]. 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*, *j* are linear, quadratic coefficients, respectively, while '*b*' is regression coefficient, *k* the number of factors studied and optimized in the experiment and 'e' is random error. 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 *F*-test. The significance of each coefficient was determined using Student's *t*-test. The coefficients of the equation and Analysis of variance (ANOVA) for the final predictive equation was done using MINITAB version 15. The response surface equation was optimized for maximum yield in the range of process variables using the MATLAB software version 7.0.1. The respective contour plots were obtained based on the effect of the levels of two parameters (at five different levels each) and their interactions on the yield of ethanol by keeping the other three 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.

Fermentation

Experiments were conducted in an online monitored modular fermenter (BIOFLO 110 Fermenter, New Brunswick Scientific Co. INC., USA) 3 litre capacity, equipped with disc impeller, oxygen and pH electrodes. The equipment also monitored temperature, agitation speed, gas purging flow rate, pumping rates, antifoam addition, dissolved oxygen (dO_2) and the vessel level. The agitation speed (200±1 rpm) and dissolved oxygen (0.05±0.1 ppm) were kept constant during the experiments. Other parameters, like substrate concentration, 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. For each experiment, 10 ml of the inoculum was used, that is, 10% (v/v) of the initial working volume (1L). The process was conducted throughout 72 hrs. Samples were withdrawn periodically (12 h interval), centrifuged in a laboratory desktop centrifuge at 1200 rpm, and the supernatants were analyzed for ethanol and residual sugar concentrations.

Analytical methods

Cellmass was determined by direct optical density at 660 nm using SYSTRONICS colorimeter (420 – 820 nm). After harvesting the cells by centrifugation at 11806x*g* (8000 rpm) for 10 min, the supernatant was used to determine the ethanol and residual sugar concentration. Ethanol was estimated using NUCON 5765 Gas Chromatography (GC) with a Flame Ionization Detector (FID) and Carbowax (2m x 0.32 cm) column using Nitrogen as the carrier gas at the rate of 40µl per minute. The oven temperature was held at 80°C. The injector and detector temperature was maintained at 200°C. Total reducing sugar concentration was determined by the Dinitrosalicylic acid (DNS) method [16] using a UV/Visible spectrophotometer ELICO BL 198 at 510 nm.

Results and Discussion

Optimization of process variables for ethanol production

Table 1 shows the four independent variables (substrate composition, initial pH, fermentation temperature, incubation time) and their concentrations at different coded and actual levels of the variables employed in the design matrix. Five level central composite design matrix and the experimental responses of the dependent variable (ethanol concentration, g/l) are listed in Table 2. Using the designed experimental data presented in Table 2, the polynomial proposed model for ethanol yield was regressed by only considering the significant terms. The expanded equation (2) is shown below.

$$Y = 14.560 + 0.917x_1 - 0.560x_3 - 1.770x_1^2 - 2.718x_2^2 - 2.575x_3^2 - 1.118x_4^2 - 0.474x_1x_2 - 0.238x_1x_3 - 0.892x_1x_4 + 0.262x_2x_4 - 0.392x_3x_4 \quad (2)$$

Based on the experimental response the quantity of ethanol produced by using *Saccharomyces cerevisiae* ranged from 3.21 to 14.56 g/l. Runs # 8 and # 27 had the minimum and maximum ethanol production respectively. The regression coefficients, along with the corresponding *P*-values, for the model of ethanol production by using *Saccharomyces cerevisiae*, are described in Table 3.

Table 1. Codes and actual levels of the independent variables for design of experiment

Independent variables	Symbols	Coded levels				
		–2	–1	0	+1	+2
Substrate conc. (g/l)	X_1	50	60	70	80	90
pH	X_2	5.0	5.5	6.0	6.5	7.0
Temperature (°C)	X_3	30	32	34	36	38
Fermentation time (h)	X_4	36	42	48	54	60

It showed that the regression coefficients of linear term *X1, X3* and all quadratic coefficients of *X1, X2, X3,* and X_4 were significant at < 1% level and interaction coefficient of X_1X_3, X_1X_4, X_2X_4 and X_3X_4 were significant at < 5% level. The *P*-values 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. The ANOVA result of quadratic regression model for ethanol yield is described in Table 3. ANOVA of the regression model for ethanol yield demonstrated that the model was significance due to an *F*-value of 144.05 (interaction effect) and a very low probability value (P model >*F* – 0.005). *F*- value several times greater than the tabulated *F*-value showed that the model predicted the experimental results well and the estimated factors effects were real. ANOVA (*F*-test) for the model explained the response of the dependent variable Y. The high F value and non-significant lack of fit indicate that the obtained experimental data is a good fit with the model. Table 2 show that the experimental yields fitted the second order polynomial equation well as indicated by high R^2 (coefficient of determination) value is 0.999 (a value > 0.75 indicates fitness of the model). The 'adjusted R^2' is 0.998, which indicates that the model is good (for a good statistical model, the R^2 value should be in the range of 0 – 1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be).

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 values				Ethanol conc. (g/l)	
	x_1	x_2	x_3	x_4	X_1 [a)]	X_2 [b)]	X_3 [c)]	X_4 [d)]	Observed	Predicted
1	-2	0	0	0	50	6.0	34	48	5.77	5.64
2	0	0	0	2	70	6.0	34	60	10.00	9.87
3	-1	1	1	-1	60	6.5	36	42	4.91	4.94
4	0	0	-2	0	70	6.0	30	48	5.34	5.38
5	0	0	0	0	70	6.0	34	48	14.56	14.56
6	2	0	0	0	90	6.0	34	48	9.22	9.31
7	-1	-1	1	1	60	5.5	36	54	4.69	4.80
8	0	0	2	0	70	6.0	38	48	3.21	3.14
9	0	-2	0	0	70	5.0	34	48	3.86	3.95
10	-1	-1	1	-1	60	5.5	36	42	4.56	4.54
11	-1	-1	-1	-1	60	5.5	32	42	4.65	4.65
12	1	1	-1	1	80	6.5	32	54	7.20	7.01
13	-1	1	-1	1	60	6.5	32	54	7.28	7.43
14	1	1	1	1	80	6.5	36	54	4.66	4.88
15	1	-1	1	1	80	5.5	36	54	5.53	5.32
16	0	0	0	0	70	6.0	34	48	14.56	14.56
17	0	0	0	-2	70	6.0	34	36	10.20	10.30
18	1	1	-1	-1	80	6.5	32	42	7.59	7.70
19	0	0	0	0	70	6.0	34	48	14.56	14.56
20	0	0	0	0	70	6.0	34	48	14.56	14.56
21	-1	1	1	1	60	6.5	36	54	6.21	6.26
22	-1	-1	-1	1	60	5.5	32	54	6.56	6.48
23	1	-1	-1	1	80	5.5	32	54	7.77	7.96
24	1	1	1	-1	80	6.5	36	42	7.27	7.14
25	0	2	0	0	70	7.0	34	48	3.54	3.41
26	1	-1	-1	-1	80	5.5	32	42	9.95	9.69
27	0	0	0	0	70	6.0	34	48	14.56	14.56
28	0	0	0	0	70	6.0	34	48	14.56	14.56
29	0	0	0	0	70	6.0	34	48	14.56	14.56
30	-1	1	-1	-1	60	6.5	32	42	4.56	4.55
31	1	-1	1	-1	80	5.5	36	42	8.56	8.63

[a)] X_1 (Substrate concentration, g/l) is calculated as: $X_1 = 70 + x_1\,(10)$
[b)] X_2 (initial pH) is calculated as: $X_2 = 6.0 + x_2\,(0.5)$
[c)] X_3 (incubation temperature, °C) is calculated as: $X_3 = 34 + x_3\,(2)$
[d)] X_4 (fermentation time, h) is calculated as: $X_3 = 48 + x_3\,(6)$

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

Model Term	Regression coefficient	Std. deviation	*t*-statistics	*P*-value
Intercept	14.56	0.05897	246.89	< 0.001
X_1	0.917	0.03185	28.794	< 0.001
X_2	-0.135	0.03185	-4.226	0.001
X_3	-0.56	0.03185	-17.57	< 0.001
X_4	-0.106	0.03185	-3.336	0.004
X_1X_1	-1.77	0.02918	-60.651	< 0.001
X_2X_2	-2.718	0.02918	-93.167	< 0.001
X_3X_3	-2.575	0.02918	-88.24	< 0.001
X_4X_4	-1.118	0.02918	-38.331	< 0.001
X_1X_2	-0.474	0.03901	-12.161	< 0.001
X_1X_3	-0.238	0.03901	-6.105	< 0.001
X_1X_4	-0.892	0.03901	-22.864	< 0.001
X_2X_3	0.126	0.03901	3.221	0.005
X_2X_4	0.262	0.03901	6.713	< 0.001
X_3X_4	-0.392	0.03901	-10.046	< 0.001
	$R^2 = 0.99$	Adjusted $R^2 = 0.99$		

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 identify from the circular or elliptical nature of the contours. The contour plots based on independent variables were obtained using the same software package (MINITAB version 15) indicated that a local optimum exists in the area experimentally investigated (Figs.1 – 3). 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. Figs. 1 – 3 show the response contour plots of the interactive effect of substrate concentration, initial pH, incubation temperature and fermentation time on ethanol production. The effect of substrate concentration and fermentation time on ethanol production, while other variables (initial pH and incubation temperature) were fixed at central level (6.0 and 34°C respectively), was shown in Fig. 1. The drastic interactions between substrate concentration and fermentation time were apparent not only from the low probability value ($P < 0.001$, Table 3), but also from the elliptical contour plot (Fig. 1). According to Fig. 1, the contours around the stationary point were elliptical and it became elongated more and more along the substrate concentration axis, which meant that a small change of the response value would require a small move along the substrate concentration axis. It was evident that the ethanol concentration steadily decreased with increasing fermentation time upto 60 h and at low substrate concentration level. While at high fermentation, the increase in the response value was negligible with as the substrate

concentration value was increased. So a lower substrate concentration and lower fermentation time enhance the ethanol yield. Since a circular contour plot indicates that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicates that the interactions between them are significant.

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	465.569	14	33.255	1000.00	< 0.001
Linear	28.406	4	7.101	291.70	< 0.001
Square	416.122	4	104.03	4000.00	< 0.001
Interaction	21.042	6	3.507	144.05	< 0.001
Residual Error	0.39	16	0.024	-	-
Lack-of-Fit	0.39	10	0.039	-	-
Pure Error	0	6	0	-	-
Total	465.959	30	-	-	-

The other pair of the independent variables incubation temperature and fermentation time showed similar significant effects ($P < 0.001$, Table 3) while keeping the third independent variables, initial substrate concentration and initial pH as constant at 60% v/v and 34°C respectively (Fig. 2). The other pair of the independent variables pH and fermentation time showed a significant effect ($P < 0.001$,Table 3) while keeping the other independent variables, substrate concentration and incubation temperature as constant at 60% v/v and 34°C respectively (Fig. 3). The result shows that as the values of process variables increased, the ethanol concentration also increased but only up to the midpoint of range of variables and thereafter the ethanol concentration decreased even though the values of variables increased. The ethanol concentration was significantly affected by pH, temperature and fermentation time where incubation temperature and initial pH producing greater effect.

The drastic interactions between incubation temperature and fermentation time were apparent not only from the low probability value ($P < 0.001$, Table 3), but also from the elliptical contour plot (Fig. 2). Since a circular contour plot indicates that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicates that the interactions between them are significant. The other pair of the independent variables pH and fermentation time showed similar effects while keeping the third independent variable, temperature as constant at 35°C (Fig. 3). The results showed that as the values of process variables increased, the ethanol concentration also increased but only up to the midpoint of range of variables and thereafter the ethanol concentration decreased even though the values of variables increased. The ethanol concentration was significantly affected by pH, temperature and fermentation time where temperature and initial pH producing greater effect. The orientation of the principal axes of the contour plots between the variables substrate concentration and fermentation time, incubation temperature and fermentation time, and initial pH and fermentation time indicated that the mutual interactions between these set of variables

had a significant effect on the ethanol production. The ethanol yield was significantly affected by the process variables such as substrate concentration, incubation temperature, initial pH and fermentation time.

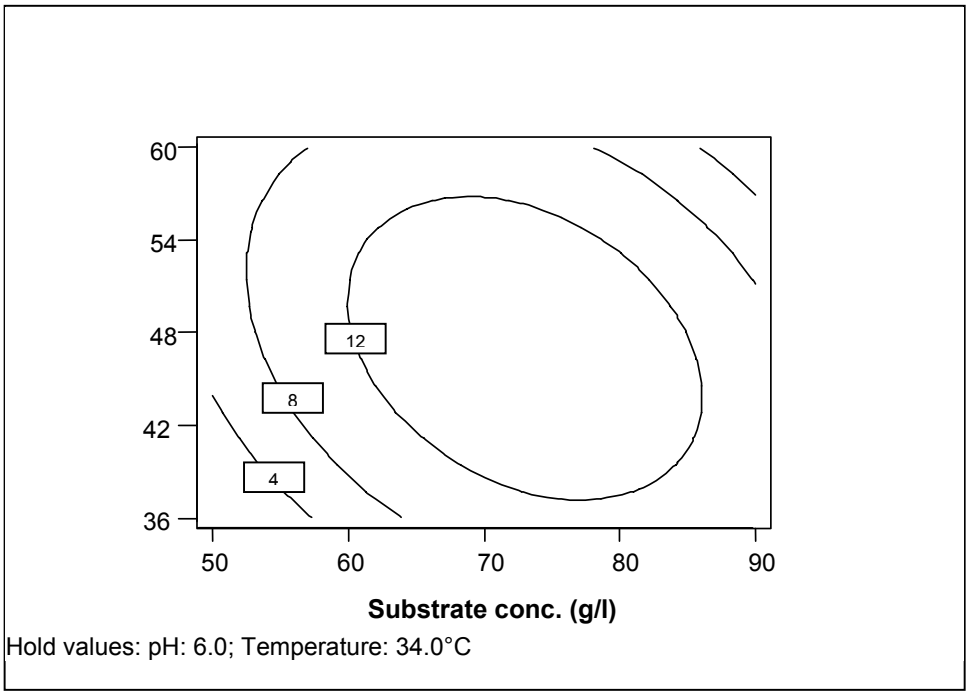

Figure 1. Isoresponse contour plot of substrate concentration versus fermentation time on ethanol production.

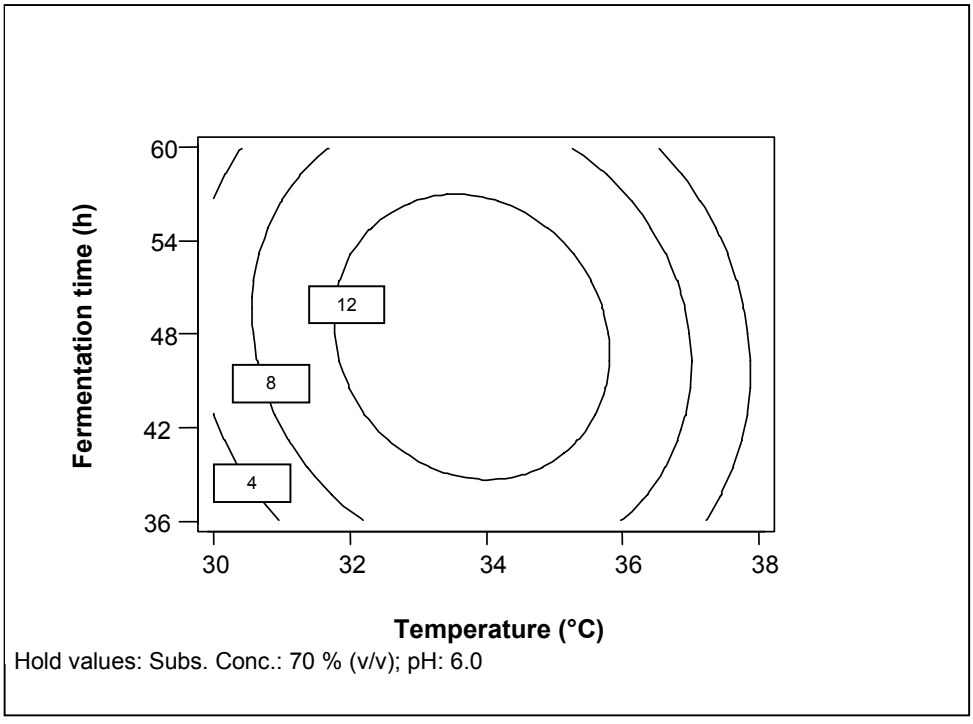

Figure 2. Isoresponse contour plot of temperature versus fermentation time on ethanol production.

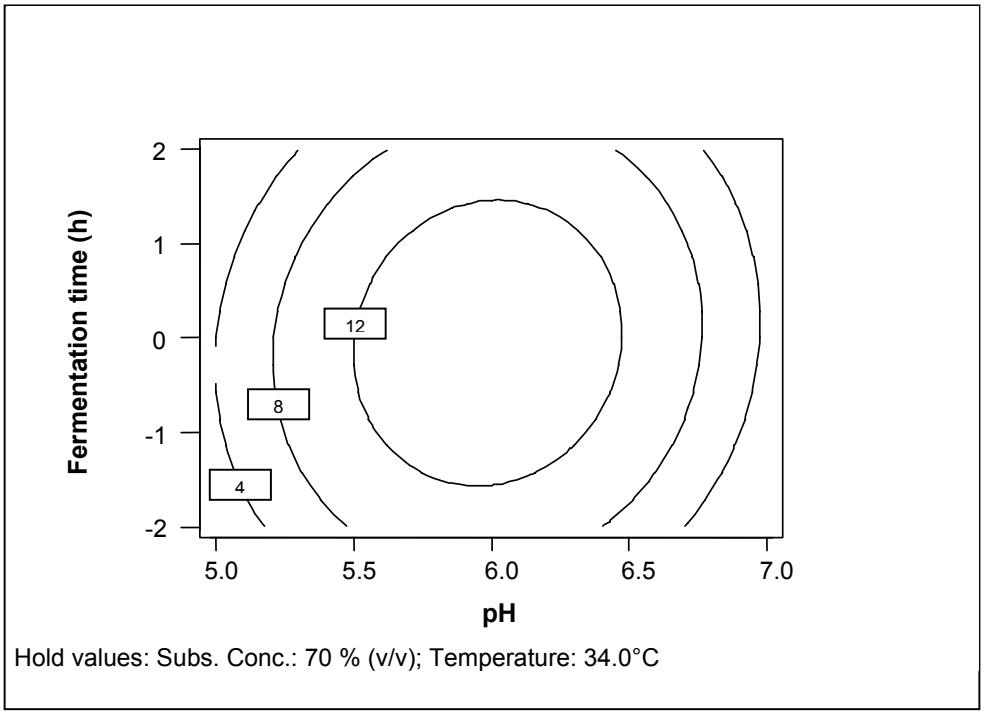

Figure 3. Isoresponse contour plot of initial pH versus fermentation time on ethanol production.

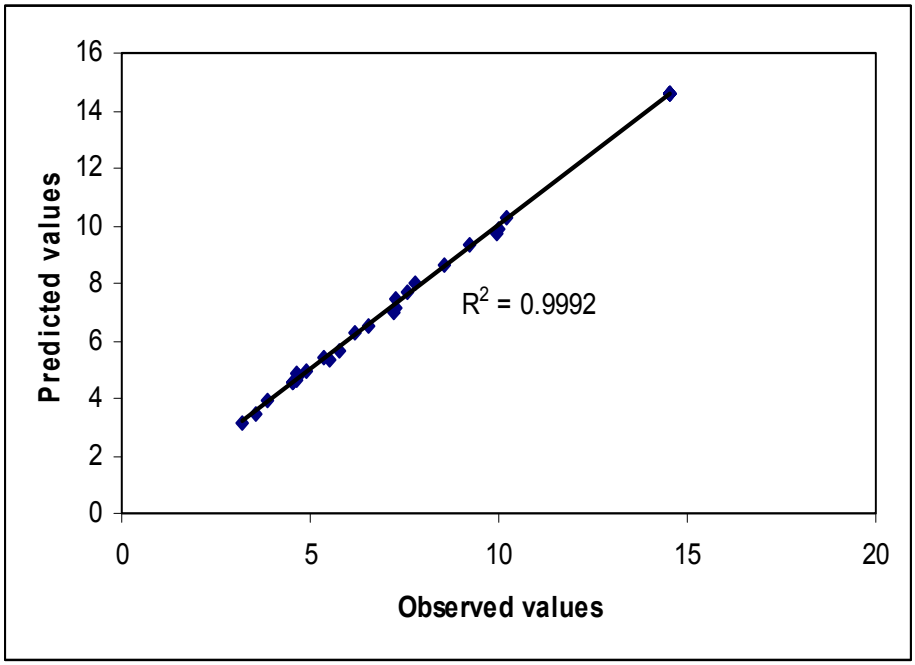

Figure 4. Parity plot showing the distribution of experimental versus predicted values by the mathematical model of the Y (ethanol conc.) values.

The matching quality, of the data obtained by the model proposed in Equation (2), was evaluated considering the correlation coefficient, R^2, between the experimental and modeled data. The mathematical adjust of those values generated a $R^2 = 0.99$, revealing that the model could not explain only 1% of the overall effects, showing that it is a robust statistical model. The parity plot shows a satisfactory correlation between the experimental and predictive values (Fig. 4). Response analysis revealed the maximum ethanol yield by using *Saccharomyces cerevisiae* could be achieved at the conditions when substrate concentration is 62 % (v/v), initial pH of growth fermentation media is 6.5; incubation temperature, 32°C and fermentation time 42 h. Under these optimum process conditions a maximum ethanol concentration of 15.64 g/l was obtained.

Conclusion

Due to the dwindling of fossil fuel resources, microbial production of biofuel from organic waste byproducts has acquired significance as a fuel for the future. This study examines the possibility of waste whole cashew apple for ethanol production. Cashew apple has no commercial use value, except for its use by rural inhabitants in the production of homemade alcoholic beverages. Conventional optimization studies are time consuming and expensive. To overcome these problems, a Central Composite Design (CCD) was used for the optimization of process conditions. From the present study, it is evident that the use of statistical process condition optimization approach, response surface methodology has helped to locate the most significant conditions with minimum effort and time. In addition, it has also proved to be useful in increasing ethanol concentration. Only 31 experiments were necessary and the obtained model was adequate ($P < 0.001$). By solving the regression equation, the optimum process conditions were determined; substrate concentration 62 % (v/v), pH 6.5, temperature 32°C, and fermentation time 37h. A maximum ethanol concentration of 15.64 g/l was obtained at the optimized process conditions.

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