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Optimization of Fermentation Parameters to Enhance the Production of Ethanol from Palmyra Jaggery Using *Saccharomyces cerevisiae* in a Batch Fermentor

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Abstract Application of statistical experimental designs for optimization of fermentation parameters to enhance ethanol production, which is an economical and renewable energy source using *Saccharomyces cerevisiae* NCIM 3090 from palmyra jaggery, was studied in a batch fermentor. Using Plackett–Burman design, impeller speed, concentrations of $CoCl_2$ and KH_2PO_4 were identified as significant variables, which highly influenced ethanol production, and these variables were further optimized using a central composite design (CCD). The ethanol production was adequately approximated with a full quadratic equation obtained from three factors and five levels of CCD. Maximum ethanol concentration of 132.56 g/l (16.8% [v/v]) was obtained for an impeller speed of 247.179 (≈250) rev/min, $CoCl_2$ of 0.263 g/l and KH_2PO_4 of 2.39 g/l. A second-order polynomial regression model was fitted and was found adequate with R^2 of 0.8952. This combined statistical approach

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enables rapid identification and investigation of significant parameters for improving the ethanol production and could be very useful in optimizing processes.

Keywords *Saccharomyces cerevisiae* · Batch fermentor · Palmyra jaggery · Ethanol · Plackett–Burman design · Central composite design

Introduction

Improvement of ethanol production has been continuing because it is an economical and renewable alternative energy source to fossil fuels and also it is one of the few bulk organic chemicals having a variety of uses as a chemical, intermediate, and solvent. Fermentative ethanol has remained significant in Belgium, Italy, France, and The Netherlands and its importance is growing in Japan [1]. Ethanol is a good transportation fuel; it can be blended with gasoline—10 to 22% blends are being used in the US and Brazil. Ethanol blended with gasoline provides a number of benefits viz., reduces the formation of carbon monoxide and ozone [2], reduces NO_2 emission from combustion, and has clean burning properties [3]. Ethanol is produced commercially by submerged fermentation using *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* has an efficient anaerobic sugar metabolism and tolerates industrial inhibitory substrates better than other microorganisms [4, 5]. Trace elements are known to have a profound effect on the growth of *S. cerevisiae* in the production of ethanol and *S. cerevisiae* requires a variety of trace metal ions in limited concentrations [6, 7]; excess concentrations result in lower yields of ethanol, causing inhibitory effect [8, 9]. The tolerance of different strains to trace elements varies widely and it is necessary to determine the optimum concentration to avoid the inhibitory effect caused when these cations are present in higher concentrations. Phosphate is essential for the growth of yeast and is usually supplied as orthophosphate salts.

Ethanol can be produced by different ways viz., batch, continuous, fed-batch, and semicontinuous processes. However, most of the ethanol is produced by batch fermentation process because it is economical [10]. In a batch process, agitation plays an important role and hence an optimum impeller speed will aid congenial environment for the fermentation. Ethanol can be successfully produced from various substrates. The suitable substrate is the main cost component for industrial ethanol production and it is essential that ethanol production should be carried out with cheap substrates [11, 12]. Palmyra jaggery, a sugar syrup from the palmyra palm (*Borassus flabellifer*) is an agricultural product available in

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India Peninsula and Northern of Sri Lanka and is an alternative renewable and suitable raw material for producing ethanol.

The first step in process optimization is the screening of important variables, followed by estimation of optimal levels of these variables [13]. Plackett–Burman design (PBD) [14] is a well-established and widely used statistical technique for screening of the important variables. After screening of the important variables the conventional method used for optimization is the "one factor at a time" method in which a single factor or one independent variable is varied while fixing all others at a specific level. This may lead to unreliable results and less accurate conclusions. This method requires us to carry out a number of experiments to determine the optimum levels and it is also time consuming. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by central composite design (CCD) [15] using response surface methodology (RSM). A detailed account of this technique has been outlined [16]. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Several researchers have applied these techniques in biotechnology for optimization of different parameters [17–19]. Hence the present study reports the application of PBD and CCD to optimize ethanol production from palmyra jaggery as the substrate in a batch fermentor.

Materials and Methods

Raw Material (Substrate)

Palmyra jaggery is the dark semisolid obtained from the sweet toddy, which is collected from the palmyra tree (*B. flabellifer*) grown in Varighedu, West Godavari District, and Andhra Pradesh, India.

Microorganism

Saccharomyces cerevisiae NCIM 3090, obtained from the National Chemical Laboratory, Pune, India, was used throughout the study.

Growth Conditions

The culture was maintained on agar slants having the following composition (% w/v): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5, and agar-agar 2.0. The pH of the medium was adjusted to 6.4–6.8 and incubated at 30 °C for 48 h.

Fermentation Conditions

Palmyra jaggery with 70% sugars was used as the sole carbon source for the fermentation. Fermentation medium was composed of 398.5 g/l of substrate (sugar syrup), 3.1 g/l of urea, and 0.51 g/l of EDTA. Submerged fermentation was carried out in a Biostat M fermentor supplied by B. Braun Co., Germany, with all necessary controls. The fermentor was of 2 l in capacity and the working volume was 1 l. Inoculum was prepared in a 500-ml flask containing 100 ml of inoculum medium by incubating it at 30 °C for 48 h on a rotary

Table 1 Variables for screening using PBD.

Compound	(+) Level	(−) Level
Peptone (g/l)	18	1.0
Yeast extract (g/l)	18	1.0
KH_2PO_4 (g/l)	6.0	1.0
K_2HPO_4 (g/l)	0.05	1.0
Na_2HPO_4 (g/l)	4.0	1.0
Impeller speed (rev/min)	600	50
$CoCl_2$ (g/l)	0.5	0.1

shaker. The ranges of variables for the optimization were maintained as follows: impeller speed of 30–400 rev/min, $CoCl_2$ of 0.1–0.5 g/l, and KH_2PO_4 of 1.5–3.5 g/l. The reactor was maintained under anaerobic conditions without aeration. The operating conditions were maintained at a temperature of 26 °C, pH of 8.4, and fermentation time of 4.2 days.

Analytical Methods

Ethanol was estimated by GC in which a flame ionization detector and a stainless steel column (2.0 m length, 3.0 mm I.D.) packed with Porapak-Q (50–80 mesh, manufactured by Nucon Engineers, India) were used. The column oven was operated isothermally at 150 °C and the detector and injection ports were kept at 170 °C. Nitrogen was used as the carrier gas at a flow rate of 30 cm^3/min and the combustion gas was a mixture of hydrogen and air [20]. Sugars were determined using Miller's method [21].

Experimental Design and Optimization

Plackett–Burman Design

The purpose of the initial optimization step was to identify the parameters, which have a significant effect on ethanol production. The Plackett–Burman statistical experimental design [13] is very useful for screening the most important variables from a lot. The total number of experiments to be carried out is $K+1$, where K is the number of variables. Each variable is represented at two levels, high and low denoted by (+) and (−), respectively. The

Table 2 Plackett–Burman experimental design matrix for screening of important variables for ethanol production.

Run no.	Variable level							
	Peptone	Yeast extract	KH_2PO_4	K_2HPO_4	Na_2HPO_4	Impeller speed	$CoCl_2$	Ethanol (g/l)
1	−	−	−	+	+	+	−	94.28
2	+	−	−	−	−	+	+	98.34
3	−	+	−	−	+	−	+	99.98
4	+	+	−	+	−	−	−	96.83
5	−	−	+	+	−	−	+	97.18
6	+	−	+	−	+	−	−	95.01
7	−	+	+	−	−	+	−	93.07
8	+	+	+	+	+	+	+	97.43

Table 3 Effects for ethanol production from the results of PBD.

Variables	Effect (E)	Standard error	t (1)	p value
Peptone	0.775	0.07	11.071	0.057346
Yeast extract	0.625	0.07	8.929	0.071006
KH_2PO_4	−1.685	0.07	−24.071	0.026432*
K_2HPO_4	−0.170	0.07	−2.429	0.248668
Na_2HPO_4	0.320	0.07	4.571	0.137101
Impeller speed	−1.470	0.07	−21.00	0.030292*
$CoCl_2$	3.435	0.07	49.071	0.012972*

*Significant at $p \leq 0.05$

statistical software package STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA) was used for analyzing the experimental data.

The effect of each variable on ethanol production was calculated by using the following equation:

$$E_{(Xi)} = \frac{\Sigma Y_{(+)i} - \Sigma Y_{(-)i}}{L/2} \quad (1)$$

where $E_{(Xi)}$ is the effect of levels of the tested variables, $Y_{(+)i}$ and $Y_{(-)i}$ are the ethanol production from trails in which the variables being tested are added to the medium at their maximum and minimum levels, respectively, and L is the number of experiments carried out. When the value of concentration effect ($E_{(Xi)}$) of the tested variable is positive, the influence of the variable is greater at high concentration, and when it is negative, the influence of the variable is greater at low concentration.

Central Composite Design

Once the variables having the greatest influence on the responses were identified, a CCD [14] was used to optimize the levels of these variables. The CCD, based on three basic principles of an ideal experimental design, primarily consists of (1) a complete 2^n factorial design, where n is the number of variables; (2) n_0 center points ($n_0 \geq 1$); and (3) two axial points on the axis of each design variable at a distance of $2^{n/4}$ from the design center.

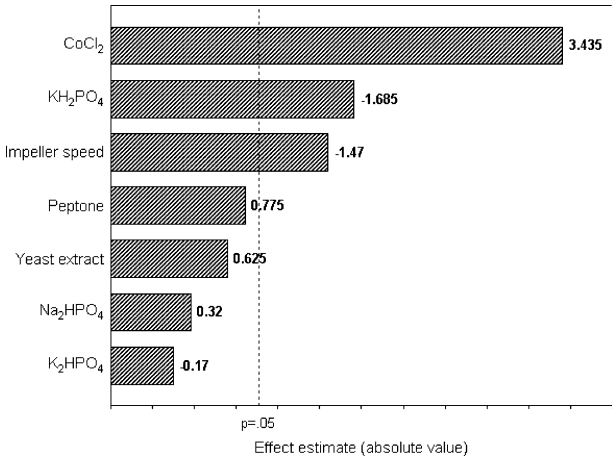

Fig. 1 Pareto graph showing effect of various variables on ethanol production based on the observations of PBD

Table 4 Independent variables in the experimental plan.

Variables	Coded levels				
	−1.682	−1	0	+1	+1.682
Impeller speed (rev/min), X_1	31.8	100	200	300	368.2
$CoCl_2$ (g/l), X_2	0.132	0.2	0.3	0.4	0.468
KH_2PO_4 (g/l), X_3	1.659	2	2.5	3	3.341

Hence, the total number of design points, $N = 2^n + 2n + n_0$. For statistical calculations the variables X_i are coded as x_i according to Eq. 2:

$$x_i = \frac{X_i - \overline{x_i}}{\Delta x_j}, (i = 1, 2, 3, \ldots \ldots \ldots \ldots \ldots, k) \tag{2}$$

where x_i is dimensionless value of an independent variable, X_i is the real value of an independent variable, $\overline{x_i}$ the is real value of the independent variable at the center point, and Δx_j is the step change.

The second-degree polynomials (Eq. 3) are calculated with the statistical package STATISTICA 6.0 (StatSoft Inc.) to estimate the response of the dependent variable:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \tag{3}$$

Table 5 The CCD matrix employed for three independent variables.

Run no.	Coded levels			Actual values			Ethanol (g/l)	
	x_1	x_2	x_3	X_1	X_2	X_3	Experimental	Predicted
1	−1	−1	−1	100	0.2	2	106.99	109.75
2	1	−1	1	300	0.2	3	116.94	117.40
3	−1	1	1	100	0.4	3	110.83	111.15
4	1	1	−1	300	0.4	2	112.36	116.06
5	0	0	0	200	0.3	2.5	128.65	129.06
6	0	0	0	200	0.3	2.5	129.72	129.06
7	−1	−1	1	100	0.2	3	106.68	107.74
8	1	−1	−1	300	0.2	2	120.81	125.24
9	−1	1	−1	100	0.4	2	107.27	111.50
10	1	1	1	300	0.4	3	107.79	109.79
11	0	0	0	200	0.3	2.5	128.92	129.06
12	0	0	0	200	0.3	2.5	129.89	129.06
13	−1.682	0	0	31.8	0.3	2.5	111.82	109.09
14	1.682	0	0	368.2	0.3	2.5	124.98	120.98
15	0	−1.682	0	200	0.132	2.5	123.69	120.79
16	0	1.682	0	200	0.468	2.5	119.78	115.93
17	0	0	−1.682	200	0.3	1.659	120.23	113.49
18	0	0	1.682	200	0.3	3.341	106.53	106.54
19	0	0	0	200	0.3	2.5	128.00	129.06
20	0	0	0	200	0.3	2.5	128.05	129.06

X_1=impeller speed (rev/min), X_2=$CoCl_2$ (g/l), X_3=KH_2PO_4 (g/l)

Table 6 Coefficients, *t* statistics, and significance probability of the model.

Term	Coefficient	Value	Standard error of coefficient	*t* value	*p* value
Constant	B_0	−110.7266	39.77809	−2.7846	0.019298
X_1	B_1	0.389319	0.093269	4.174174	0.001905*
X_2	B_2	248.2379	99.674	2.490498	0.031963*
X_3	B_3	133.9745	23.51545	5.697297	0.000199*
$X_1 \times X_1$	B_{11}	−0.0005	0.000105	−4.70449	0.000836*
$X_2 \times X_2$	B_{22}	−379.069	105.5848	−3.59019	0.004928*
$X_3 \times X_3$	B_{33}	−26.9283	4.215266	−6.38827	7.95E-05*
$X_1 \times X_2$	B_{12}	−0.27538	0.141473	−1.94649	0.080214
$X_2 \times X_3$	B_{23}	7.925	28.29456	0.280089	0.785118
$X_3 \times X_1$	B_{31}	−0.02923	0.028295	−1.03288	0.325991

Coefficient of correlation (R)=0.9461; coefficient of determination (R^2)=0.8952

*Significant at $p \leq 0.05$

where Y is predicted response; X_1, X_2, and X_3 are independent variables; b_0 is an offset term; b_1, b_2, and b_3 are linear effects; b_{11}, b_{22}, and b_{33} are squared effects; and b_{12}, b_{23}, and b_{13} are interaction terms.

Results and Discussion

Identification of Important Variables Using PBD

A total of seven variables were screened through eight experimental runs. The experimental plan and corresponding ethanol production were shown in Tables 1 and 2. Each variable is represented at two levels, with high and low denoted by (+) and (−), respectively. The results in Table 2 shows that run number "3" gave the maximum yield followed by run numbers "2" and "8." The results in Table 3 shows the resulting effects (which are calculated according to Eq. 1 as discussed earlier) of the variables on the responses, the associated *t* values, and significant levels. A *p* value less of than 0.05 for the three variables viz., impeller speed (X_1), $CoCl_2$ (X_2), and KH_2PO_4 (X_3), indicates that they are significant. The same is confirmed from the Pareto graph (Fig. 1), which was also used to show the

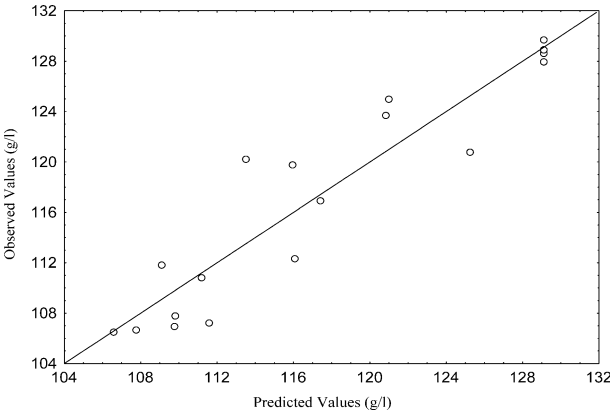

Fig. 2 Parity plot showing the distribution of predicted values vs observed values of ethanol concentration

Table 7 ANOVA for the entire quadratic model.

Source of variation	Sum of squares (SS)	Degree of freedom (*df*)	Mean squares (MS)	*F* value	Probe>*F*
Model	1366.976	9	151.8862		
Error	160.465	10	16.0465	9.465	0.000787
Total	1527.44	19			

R=0.9461; R^2 =0.8952; adjusted R^2 =0.80083

$F_{0.01(9,10)} = S_r^2 / S_e^2 = 9.465 > F_{0.01(9,10)\text{Tabular}} = 4.94$

$P_{\text{model}}>F$=0.000787

effect of all variables on ethanol production. These variables had confidence level above 95% in comparison to other variables and thus were considered to be highly significant for ethanol production. These significant variables were further optimized by CCD involving RSM.

Optimization of the Selected Variables Using CCD

Based on the results of the PBD, impeller speed (X_1, rev/min), $CoCl_2$ (X_2, g/l), and KH_2PO_4 (X_3, g/l) were chosen as the independent input variables and ethanol yield (Y, g/l) was used as the dependent output variable. A CCD was employed to analyze the interactive effect of these variables and to arrive at an optimum. A summary of the independent variables and their levels was given in Table 4. A 2^3 factorial central composite experimental design, with six axial points $(\alpha = \sqrt{3})$ and six replications at the center points (n_0=6) leading to a total number of 20 experiments (Table 5), was employed for the optimization of the parameters.

The results were analyzed using the analysis of variance (ANOVA) and χ^2 test as appropriate to the experimental design was performed. By applying multiple regression

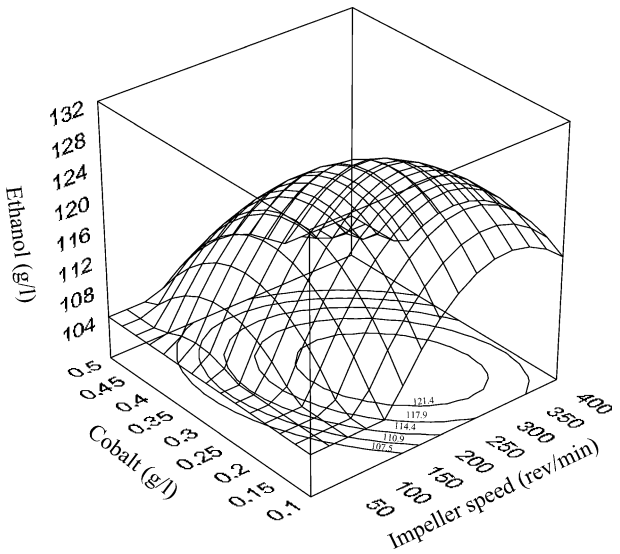

Fig. 3 Response surface and contour plot of impeller speed vs cobalt concentration on ethanol production (phosphate concentration was kept constant at 2.5 g/l)

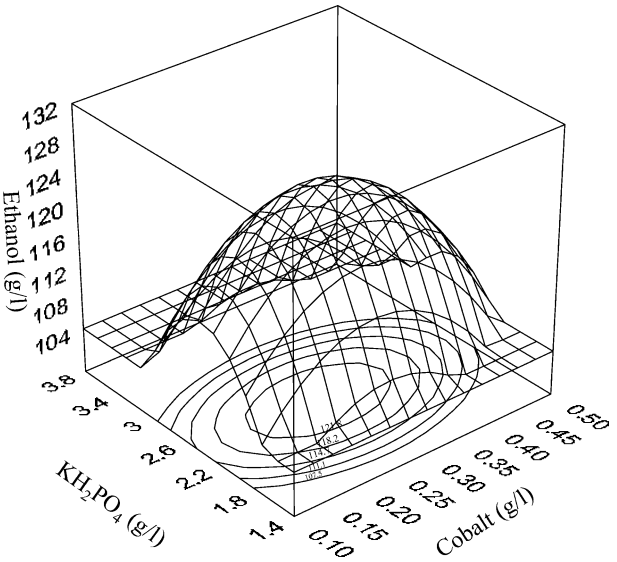

Fig. 4 Response surface and contour plot of cobalt concentration vs phosphate concentration on ethanol production (impeller speed was kept constant at 200 rev/min)

analysis to the experimental data, the following second-order polynomial equation was found to represent the ethanol production adequately:

$$Y = -110.7266 + 0.389319X_1 + 248.2379X_2 + 133.9745X_3 - 0.0005X_1^2 - 379.069X_2^2 - 26.9283X_3^2 - 0.27538X_1X_2 + 7.925X_1X_3 \quad (4)$$

The predicted values of ethanol production from palmyra jaggery medium using the above equation are given in Table 5, along with experimental data. The coefficients of the regression model (Eq. 4) calculated are listed in Table 6, in which they contain three linear, three quadratic, and three interaction terms and one block term. The significance of each coefficient was determined by Student t test and p values, which are also listed in Table 6.

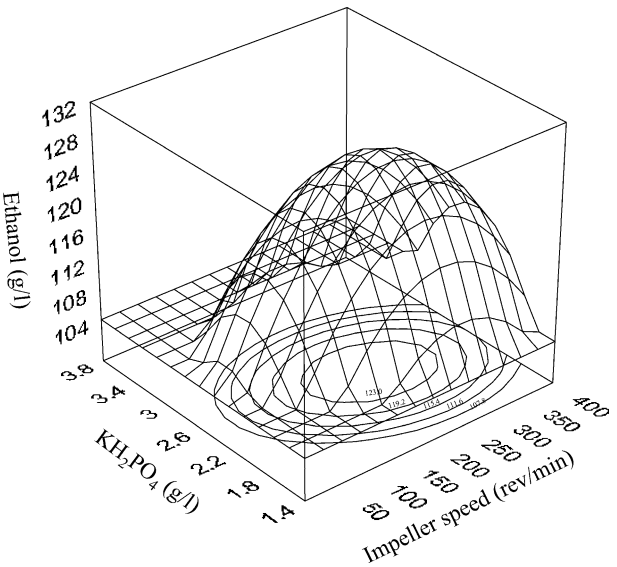

Fig. 5 Response surface and contour plot of impeller speed vs phosphate concentration on ethanol production (cobalt concentration was kept constant at 0.2 g/l)

Table 8 Optimum values of fermentation parameters and the experimental and predicted ethanol production.

Variables	Optimum values	Ethanol (g/l)	
		Experimental	Predicted
Impeller speed (rev/min), X_1	247.179		
$CoCl_2$ (g/l), X_2	0.263	132.56	133.7
KH_2PO_4 (g/l), X_3	2.39		

The larger the magnitude of the t value and the smaller the p value, the more significant is the corresponding coefficient [22, 23]. This implies that the linear and quadratic effects of impeller speed, $CoCl_2$, and KH_2PO_4 are highly significant, as is evident from their respective p values. This indicates that they can act as limiting nutrients and small variations in their concentration will alter either growth rate or product formation rate or both to a considerable extent. All the interaction terms of impeller speed, $CoCl_2$, and KH_2PO_4 are found to be insignificant ($p>0.05$). The parity plot (Fig. 2) shows a satisfactory correlation between the experimental and predicted values of ethanol production, wherein the points cluster around the diagonal line, which indicates the good fit of the model because the deviation between the experimental and predicted values is less.

The results of the second-order response surface model fitting in the form of ANOVA are given in Table 7. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F value $F\text{value} = S_r^2/S_e^2$, is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the F value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F test ($F_{\text{model}}=9.465$) and a very low probability value ($P_{\text{model}}>F=0.000787$). Moreover, the computed F value $\left(F_{0.01(9,10)}=S_r^2/S_e^2=9.465\right)$ is greater than the tabular F value ($F_{0.01(9,10)\text{Tabular}}=4.94$) at the 1% level, indicating that the treatment differences are significant.

The goodness of the fit of the model was checked by the determination coefficient (R^2). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response [16]. In this case, the value of the determination coefficient ($R^2=0.8952$) indicates that 89.52% of the sample variation in the ethanol production is attributed to the independent variables. The R^2 value also indicates that only 1% of the variation is not explained by the model. In addition, the value of the adjusted determination coefficient (adj $R^2=0.80083$) is also very high to advocate for a high significance of the model. Also, a higher value of the correlation coefficient ($R=0.9461$) justifies an excellent correlation between the independent variables [24]. Chi-square test was also carried out to check the best fit of the model. Because χ^2_{cal} is 1.4 and χ^2_{tab} is 30.14 and $\chi^2_{\text{cal}}<\chi^2_{\text{tab}}$, the model was a good fit. The predicted optimum levels of impeller speed, $CoCl_2$, and KH_2PO_4 were obtained by applying the regression analysis to the Eq. 4.

Figures 3, 4 and 5 represent the response surface and contour plots for the optimization of fermentation parameters of ethanol production. The effects of the impeller speed and $CoCl_2$ on the ethanol production are showed in Fig. 3. An increase in the impeller speed

with $CoCl_2$ up to the optimum point increased the ethanol production to a maximum level and in a further increase in the impeller speed with $CoCl_2$ the trend was reversed. The interaction effect of the $CoCl_2$ and KH_2PO_4 on the ethanol production in Fig. 4 clearly indicated a proper combination for production of ethanol. An increase in the $CoCl_2$ with KH_2PO_4 increased the ethanol production gradually but at a higher $CoCl_2$ and KH_2PO_4 the trend was reversed. The optimum for maximum ethanol production lay near the center point of the $CoCl_2$ and KH_2PO_4. A similar effect on the response was observed for the impeller speed at any level of the KH_2PO_4; an increase in the impeller speed with KH_2PO_4 up to the optimum point increased the ethanol production to maximum level and a further increase in the impeller speed with KH_2PO_4 decreased the ethanol production, as shown in Fig. 5.

The optimal values of the variables, in coded units, are as follows: x_i=0.47179, x_2=0.37, and x_3=0.22 with the corresponding Y=133.7 g/l of ethanol. The actual values of the variables, obtained by putting the respective values of x in Eq. 2, are as follows: 247.179 (~250) rev/min of impeller speed, 0.263 g/l of $CoCl_2$, and 2.39 g/l of KH_2PO_4. These results indicated that a high ethanol production was obtained with a combination of impeller speed, $CoCl_2$, and KH_2PO_4. Experimental and predicted ethanol production at the optimum levels of fermentation parameters were also determined (Table 8). A maximum ethanol production of 132.56 g/l (16.8%, v/v) was obtained at these optimum parameters. The above results proved that the statistical experimental designs using RSM could be effectively used to optimize various parameters for improving the ethanol production.

Conclusion

The present study involves the use of statistical experimental designs to optimize the parameters for maximizing the ethanol production from palmyra jaggery using *S. cerevisiae* NCIM 3090 in a batch fermentor. Three variables viz., impeller speed, concentrations of $CoCl_2$, and KH_2PO_4, were identified by PBD as significant for ethanol production. These variables are further optimized using CCD involving RSM. Among the three variables tested for the correlation between their concentrations and the production of ethanol, all the three variables showed significant influence on the production. The significant interactions between the three variables were also observed from the contour plots. The maximum ethanol obtained from palmyra jaggery was predicted to be 133.7 g/l (16.9%, v/v). The optimum conditions were found to be impeller speed of 247.179 (≈250) rev/min, $CoCl_2$ of 0.263 g/l, and KH_2PO_4 of 2.39 g/l. Thus, the present study enables us to obtain the maximum production of ethanol from a cheap and renewable substrate, so that this ethanol can be used continuously for fuel blending and for production of other chemicals.

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