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Research Article

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Optimization of Fermentation Parameters for Bioconversion of Corn to Ethanol Using Response Surface Methodology

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Abstract

The world is facing the problem of petroleum crisis and is in need of some immediate resolutions. The perception of sustainable development as a means to integrate the environmental, social and economic objectives of the society has been greatly developed in order to maximize human well-being in the present system without compromising the ability of future generations. Development that is not sustainable will inevitably lead to negative social, economic and environmental repercussions (OECD 2001). Energy is the crucial need of mankind and in addition is the priceless gift offered by the nature. The continuous rise in the use of fossil fuel and the extinguishing petroleum stocks have led us to rethink about the use of renewable energy sources that will also reduce carbon dioxide (CO_2) emissions. Bio-fuels, such as ethanol, are generally considered renewable since the CO_2 emitted into the atmosphere is recaptured by the growing crop in the next growth cycle. The most important issues relevant to the conversion of carbohydrates to ethanol are the cost and availability of substrate. Consequentially it is worthy to develop an economical process which allows the use of cheap substrates for successive conversion to ethanol. Hence, there is still need for cutting edge research to be done on an effective, economical, and efficient conversion process. The present study was conducted to optimize the ethanol production potential of maize (*Zea mays*). In order to achieve maximum ethanol production the experiments were conducted by optimizing three fermentation variables i.e. pH, temperature and substrate concentration which were optimized at different conditions using Response Surface Methodology (RSM) by design-expert software (version 8. 0.7.1 Stat-Ease Inc; USA). Parameters were optimized by central composite design observing the effect of combination of two variables and keeping the one constant on ethanol production. During the experiments, the maximum ethanol production was 74.6 g/L at conditions: pH 5.8, temperature 31°C and substrate concentration 160 g/L. The RSM is a better method for optimization of parameters as it is less labor intensive and more accurate than the classical methods. It reduces the number of fermentation batches. The adequacy of all the models was found to be significant at 99% as coefficients of determination were found to be (0.9923) (0.9735) (0.9662).

Keywords: Biofuels; Dry grind process; Ethanol; Fermentation; Response surface methodology

Introduction

Energy is vital need to sustain life. The demand for fossil fuels is continuously increasing but at the same time and petroleum stocks are extinguishing. This problem has led us to move around about the use of renewable energy sources. These renewable sources reduce carbon dioxide (CO_2) emissions. At present, biofuels come out to be used as the best possible economical substitute for petroleum-based fuels. Bio-fuels, such as ethanol, are renewable because the CO_2 released into the atmosphere is recaptured in the next growth cycle by the growing crop. The most primitive organic reaction that man learned to carry out is the fermentation of sugar into ethanol. The man-made ethanol is known from ancient times and its history is very long. Ethanol is very potent psychoactive substance and is used in history as a recreational drug. Ethanol, both liquor and a fuel, has been around in the form of Moonshine Whiskey in Scotland since 15th Century. In the year 1796, Johann Tobias Lowitz obtained pure ethanol by filtering distilled ethanol through activated charcoal. Ethanol is not a novel fuel as it was used as a major lighting fuel in 1850s. To raise money for the war, a liquor tax was imposed on ethanol during the Civil War. As a result, the tax increased the price of ethanol to a great extent that it could no longer compete with other fuels such as kerosene in lighting devices. This tax declined the ethanol production sharply and production levels did not begin to recover until the tax was repealed in 1906.

The cost and availability of substrate are the most important issues relevant to the conversion of carbohydrates to ethanol. For the long term planning various cellulosic substances appear to be striking as raw materials but currently are not competitive as substrates for ethanol production. Starchy materials, however, have been proposed and have proven viable as a substrate for ethanol production. The key to success of conversion of starch into sugars is the availability of highly active enzymes, suitable strain and the optimization conditions of substrate concentration, temperature and pH. More than a decade ago USA began a hard line search to find a practical source of renewable fuel to meet its voracious energy demands. Alternative fuels such as starch-based ethanol, cellulosic ethanol, and biodiesel are all considered to be potential solutions in a national effort to reduce gasoline usage by 20 percent over the next ten years [1,2]. Corn-based ethanol emerged as an early leader due to the abundance of corn and the popularity of ethanol-gasoline mixtures. The Energy Tax Act of 1978 created ethanol tax credits in an effort to decrease USA's susceptibility to oil. Between 1979 and 1986, domestic production of ethanol rose considerably in the

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USA, from a mere 20 million US liquid gallons (over 75 million liters) to 750 million gallons (around 2.84 billion liters) [3]. In 1990, small-scale producers received an additional tax credit of 10 cents per gallon. By 2004, the US ethanol production had grown even more. The Energy Policy Act of 2005 was another important step in corn ethanol history. It mandated an annual consumption of 7.5 billion gallons of ethanol by 2012. Two years later, the mandate was increased to 15 billion gallons of corn ethanol by 2015.

The present study was accomplished using Response Surface Methodology (RSM). It is a major process optimization tool that is used to determine the optimum values of a variety of factors significant for the process. RSM is a compilation of statistical techniques to design experiments, evaluate the effects of variables and thereby, seeking the optimum conditions. It is mostly used in optimization of different types of fermentations and bioprocesses [4-6]. The major advantage of RSM is the confined sets of experimental runs that are required to provide sufficient information for statistically acceptable results, in addition, its suitability for multiple factor experiments and examination of common relationship between various factors under experiment towards finding the most suitable production conditions for the bioprocess and forecast response. This is a group of techniques that are used to study the reaction between one or more measured dependent factors (responses) and a number of input (independent) factors.

Materials

- **Maize genotypes:** Genetically pure seeds of seven single cross hybrids of maize were procured from Directorate of Maize Research, IARI campus, PUSA, New Delhi.
- **Fermenting Yeast:** The fermenting yeast (*Saccharomyces cerevisiae* MTCC 4043) used in the present study was procured from IMTECH, Chandigarh.
- **Commercial Enzymes:** Alpha amylase was procured from HI-MEDIA chemicals having activity1:2000 I.P.Units. Glucoamylase was procured from SRL, New Delhi. The enzyme has activity 64 units/mg.
- **Fermentor:** An applikon fermentor having capacity 3 L was used in the experiment.
- **Distillation unit:** A fractional distillation apparatus was used in the present study.

Experimental set-up

The experiments were conducted by making combinations of the variables using two factorial composite design of RSM [7]. Initially, the pH and temperature effects were observed on ethanol concentration. Nine experiments for fermentation were conducted using Central Composite Design (CCD) (design expert software). Further, the effect of temperature and substrate concentration was observed on ethanol concentration using the same method. Again, nine experiments were conducted by CCD (design expert software) for fermentation batches. Finally, the effect of pH and substrate concentration on ethanol production was observed. Subsequently, nine experiments were conducted using CCD (design expert software) performed for fermentation.

RSM is a three factorial design where contour plots are generated by linear or quadratic effects of key factors and a model equation is derived that fits the experimental data to calculate optimal responses of the system. To calculate optimum values of three factors (pH, temperature and substrate concentration) selected for CCD. An optimization study seeks a solution to an objective (minimization and maximization of an analysis feature parameters) while being constrained by a set of model dimensions and other analysis feature parameters. In order to maximize the ethanol production effective factors, the levels were selected based on previous studies. The selected variables include pH, temperature and substrate concentration. High (+) and low (-) values of these three variables were examined. A Central Composite Design was constructed which gave different values in the form of a matrix, for the selected variables.

Optimization by central composite rotable design

For the optimization of process parameters, statistical experimental design advance approach was used to provide information on the interactive effect of variables; finally, verification of experiments is used to validate the results under specific experimental conditions [8]. The influence of temperature, pH and substrate concentration on ethanol production was determined using RSM. The results of two level factorial experiment designs with five replications of the central point and six axial points are summarized in Tables 1-3 with alpha value of 1.414 and 13 runs. The effect of each factor and their interactions were analyzed using the analysis of variance (ANOVA). RSM is being widely

Run	Factor 1 A: pH	Factor 2 B: Temp (^{0}C)	Response 1 Ethanol (g/L)
1	7.2	31.00	36
2	5.8	31.00	76.4
3	5.8	18.27	52
4	5.8	43.73	47
5	4.8	40.00	57.8
6	4.3	31.00	59.7
7	6.8	22.00	44.7
8	4.8	22.00	58.2
9	6.8	40.00	42

Table 1: The combined effect of two factors i.e. pH and temperature in 9 combinations. Standard order 2 represents the maximum ethanol concentration.

Run	Factor 1 A: temp (^{0}C)	Factor 2 B: Substrate (g)	Response 1 Ethanol (g/L)
1	22.00	140.00	57
2	31.00	188.28	61
3	18.27	160.00	52
4	40.00	140.00	53.5
5	31.00	160.00	74.6
6	43.73	160.00	47
7	31.00	131.72	68
8	40.00	180.00	50
9	22.00	180.00	55

Table 2: The combined effect of two factors i.e. temperature and substrate concentration in 9 combinations. Standard order 5 represents the maximum ethanol concentration.

Run	Factor 1 A: pH	Factor 2 B: Substrate (g)	Response 1 Ethanol (g/L)
1	5.80	160.00	74.6
2	5.80	188.28	61
3	4.80	180.00	57
4	7.21	160.00	36
5	6.80	140.00	48.2
6	4.39	160.00	59.7
7	6.80	180.00	43
8	5.80	131.72	68
9	4.80	140.00	60.1

Table 3: The combined effect of two factors i.e. pH and substrate concentration in 9 combinations. Standard order 1 represents the maximum ethanol concentration.

used in optimizing different types of fermentations and bioprocess. RSM is applied to evaluate the effect of pH, temperature and substrate concentration on ethanol production by making combination of variables.

Central composite design (CCD) is the most common experimental design used in RSM, and the design exhibits equal certainty in all directions from the center. The F-test analysis of variance (ANOVA) was used to check the statistical significance of model equation. Experimental data was analyzed via response surface methodology in order to fit the following second order polynomial equation generated by Design Expert software (Trial VERSION 8.0.7.1 Stat-Ease Inc; USA). Second order coefficients were generated via regression. The response was initially fit to the factors via multiple regressions. The quality of the fit of the three models was evaluated using the coefficients of determination and analysis of variance. The three quadratic response surface models conducted by Central composite design were fit to the following equations:

I. $Y = +76.40 - 7.85A - 1.25B - 0.57AB - 13.78A^2 - 12.95B^2$

Where, A=Temperature, B=pH, Y=Ethanol concentration

II. $Y = +74.60 - 1.95A - 1.92B - 0.37AB - 13.33A^2 - 5.83B^2$

Where, A=Temperature, B=Substrate concentration, Y=Ethanol concentration

III. $Y = +74.60 - 7.43A - 2027B + 0.53AB - 14.47A^2 - 6.07B^2$

Where, A=pH, B=Substrate concentration, Y=Ethanol concentration.

Process methodology

Maintenance of culture: *Saccharomyces cerevisiae* MTCC 4043 was maintained by subculturing it every 15-20 days on YEPD agar plates and incubated in a BOD incubator at 30°C, pH 5.4 for 24 hours.

Inoculum preparation and inoculation: The inoculum for yeast was prepared in YEPD liquid medium. A loop full of strain MTCC 4043 was inoculated in the liquid medium. The culture was incubated in a BOD incubator at 30°C 150 rpm for 24 hours. This inoculum was used 10% in the sterilized corn mash.

Process of conversion of corn to ethanol: In the present study, ethanol is made through dry milling process [9,10]. However, ethanol can be made both by dry and wet milling of corn. In dry milling the entire corn kernel is processed without separating out the various component parts of the grain such as the germ. Water is added to form the slurry to which the enzymes are added to convert the starch to dextrose, a simple sugar. It is cooked at high temperatures to reduce bacterial levels, and then it is cooled and transferred to fermenters where yeast is added to convert the sugars to ethanol. Cooking and fermentation converts the starch in the grain to sugar and ethanol [11]. Left behind is the 'stillage'-comprising of protein as well as fiber. This stillage is sent through the centrifuge that separates the solid matter and soluble which are concentrated to syrup of 30% solids by evaporation. The solid matter and syrup are combined and dried together to produce a co-product called distiller's dry grain with soluble (DDGS). DDGS is a high quality, medium protein, nutritious feed ingredient widely used in beef and dairy cattle, poultry and swine feed.

Grinding

The whole corn kernel was grinded to very fine powder.

Fermentation

The substrate was dissolved in 1000 ml distilled water in the fermentation vessel. The vessel was autoclaved along with slurry. When the temperature of fermentation vessel after autoclaving reaches to 60-70°C, α-amylase [12] was added in the fermentation vessel. The pH, temperature and substrate concentration of the vessel was set in accordance with different matrix design from RSM (Tables 1-3). After 5 hrs, glucoamylase along with the 24 hrs old culture of *S. cerevisiae* MTCC 4043 was added in the vessel. The fermentation cycle was run for 72 hrs. The sample was filtered and distilled after 72 hrs.

Results and Discussion

Matrix designs of combination of variables conducted by central composite analysis: Tables 1-3 describe the results of two level factorial experiment designs with five replications of the central point and six axial points.

ANOVA for Response Surface Quadratic Models

Analysis of variance of quadratic model (i)

ANOVA and regression coefficients are listed in Table 4. The model F value of 310.89 implies that model is significant (with only 0.01% chance that the value could occur due to noise). Values of probability less than 0.050 indicate model terms are significant. In this case linear factors (A,B), quadratic factors (A^2,B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model. Both the quadratic and linear effect of temperature and pH are significant. The effect of pH is more significant than temperature. These data analysis also validate the inference that can be drawn from 3-D contour plots as shown in Figure 1 which represents the effect of pH and temperature on ethanol production. The "Predicted R-Squared" of 0.9681 is in reasonable agreement with the "Adjusted R-Squared" 0.9923 (Table 5)."Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 43.110 indicates an adequate signal (Table 5). This model can be used to navigate the design space.

Source	Sum of Squares	df	Mean Square	F Value	P Value Prob<F
Model	2707.84	5	541.57	310.89	<0.0001 significant.
A-pH	493.24	1	493.24	283.15	<0.0001
B-temp	12.93	1	12.93	7.42	0.0296
AB	1.32	1	1.32	0.76	0.4125
A^2	1320.00	1	1320.00	757.76	<0.0001
B^2	1166.63	1	1166.63	669.71	<0.0001
Residual	12.19	7	1.74		
Lack of Fit	12.19	3	4.06		
Pure Error	0.000	4	0.000		
Core Total	2720.03	12			

Table 4: Analysis of variance of quadratic model 1 (Partial sum of squares type III).

Std. Dev.	1.32	**R-Squared**	0.9955
Mean	59.95	**Adj R-Squared**	0.9923
C.V. %	2.20	**Pred R-Squared**	0.9681
PRESS	86.71	**Adeq Precision**	43.110

Table 5: Standard deviation and correlation coefficients.

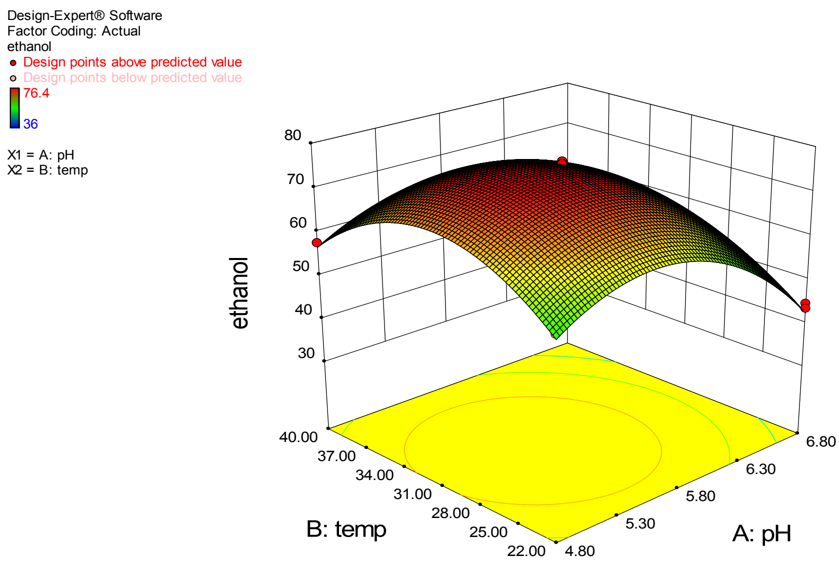

Figure 1: Response surface and contour plot showing the effect of pH and temperature on ethanol production.

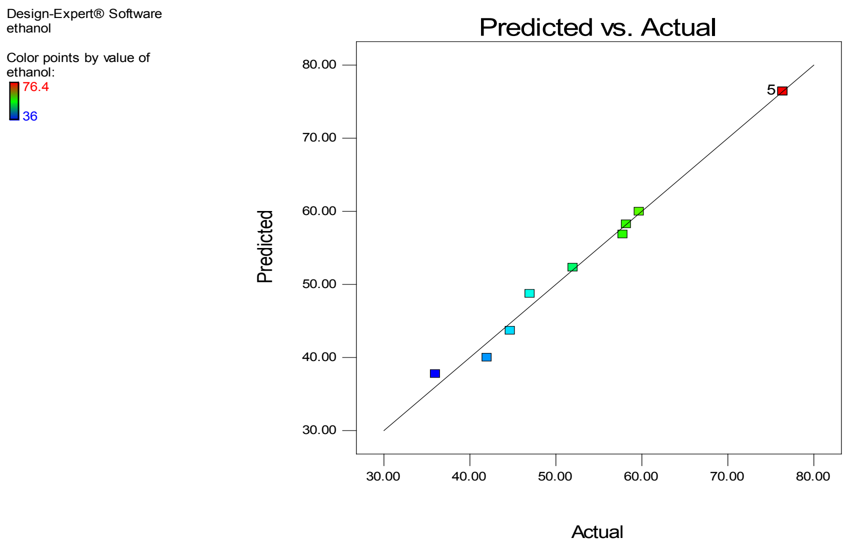

Figure 2: Predicted *vs.* Actual response of experimental run under Central Composite Design.

Source	Sum of Squares	df	Mean Square	F Value	P Value Prob<F
Model	1415.36	5	283.07	89.23	<0.0001 significant.
A-temp	30.31	1	30.31	9.55	0.0175
B-substrate	29.64	1	29.64	9.34	0.0184
AB	0.56	1	0.56	0.18	0.6863
A^2	1236.33	1	1236.33	389.73	<0.0001
B^2	236.55	1	236.55	74.57	<0.0001
Residual	22.21	7	3.17		
Lack of Fit	22.21	3	7.40		
Pure Error	0.000	4	0.000		
Core Total	1437.57	12			

Table 6: Analysis of variance of quadratic model (Partial sum of squares type III).

Analysis of variance of quadratic model (ii)

ANOVA and regression coefficients are listed in Table 6. The model F value of 89.23 implies that model is significant (with only 0.01% chance that the value could occur due to noise). Values of probability less than 0.050 indicate model terms are significant. In this case both the linear factors A, B and quadratic factors (A^2,B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model Figure 2. The quadratic effect of temperature and substrate is more prominent than linear effect. It can also be concluded from the table that effect of temperature is more pronounced than substrate concentration. These data analysis also validate the inference that can be drawn from 3-D contour plots as shown in Figure 3 which represents the effect of pH

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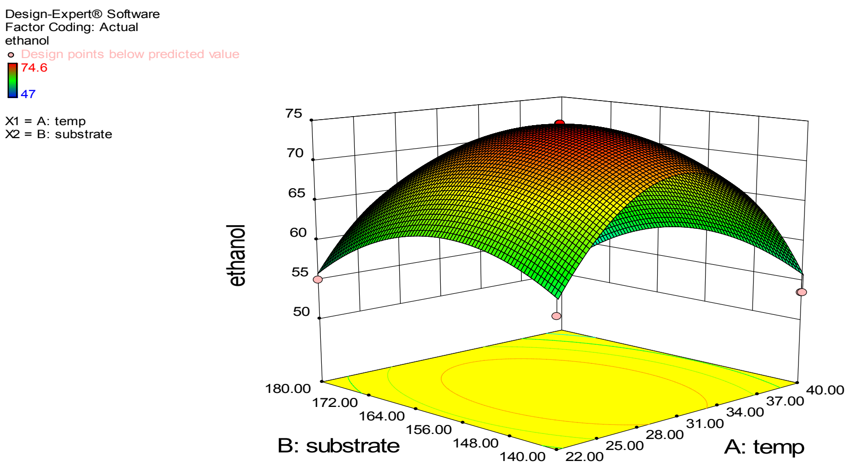

Figure 3: Response surface and contour plot showing the effect of temperature and substrate concentration on ethanol production.

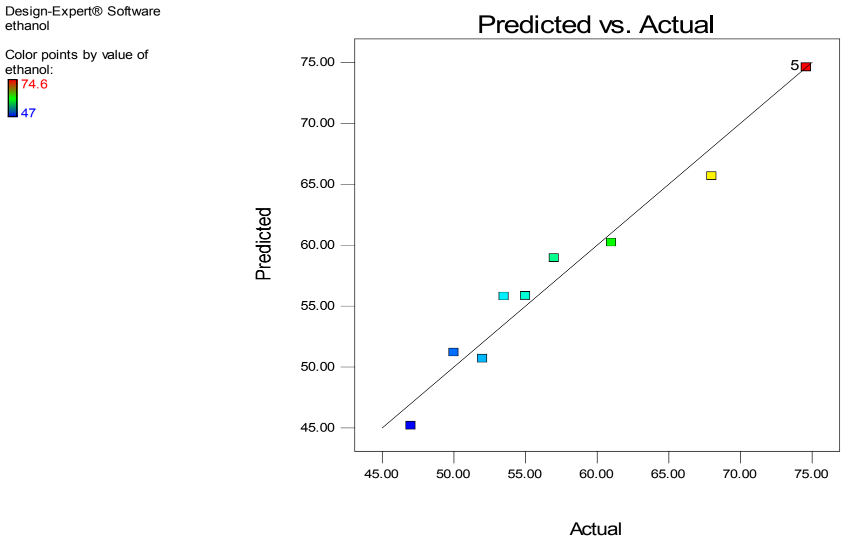

Figure 4: Predicted *vs.* Actual response of experimental run under Central Composite Design.

Std. Dev.	1.78	**R-Squared**	0.9846
Mean	62.81	**Adj R-Squared**	0.9735
C.V. %	2.84	**Pred R-Squared**	0.8902
PRESS	157.91	**Adeq Precision**	24.310

Table 7: Standard deviation and correlation coefficients.

and temperature on ethanol production. The "Predicted R-Squared" of 0.8902 is in reasonable agreement with the "Adjusted R-Squared" 0.9735."Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 24.310 indicates an adequate signal (Table 7). This model can be used to navigate the design space.

Analysis of variance of quadratic model (iii)

ANOVA and regression coefficients are listed in Table 8. The model F value of 69.71 implies that model is significant (with only 0.01% chance that the value could occur due to noise). Values of probability less than 0.050 indicate model terms are significant. In this case both the linear factors A, B and quadratic factors (A^2,B^2) are significant terms. Values greater than 0.100 indicate model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model rejection may improve the model (Figure 4).

Source	Sum of Squares	df	Mean Square	F Value	P Value Prob<F
Model	2050.95	5	410.19	69.71	<0.0001 significant.
A-pH	441.30	1	441.30	74.99	0.0001
B-substrate	41.40	1	41.40	7.04	0.0328
AB	1.10	1	1.10	0.19	0.6782
A^2	1442.50	1	1442.50	245.13	<0.0001
B^2	256.73	1	256.73	43.63	0.0003
Residual	41.19	7	5.88		
Lack of Fit	41.19	3	13.73		
Pure Error	0.000	4	0.000		
Core Total	2092.14	12			

Table 8: Analysis of variance of quadratic mode (Partial sum of squares Type III).

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The quadratic effect of temperature and substrate is more prominent than linear effect. It can also be concluded from the table that effect of temperature is more pronounced than substrate concentration. These data analysis also validate the inference that can be drawn from 3-D contour plots as shown in Figure 5 which represents the effect of pH and temperature on ethanol production. The "Predicted R-Squared" of 0.8600 is in reasonable agreement with the "Adjusted R-Squared" of 0.9662. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 23.849 indicates an adequate signal (Table 9). This model can be used to navigate the design space (Figure 6).

Std. Dev.	2.43	**R-Squared**	0.9803
Mean	62.00	**Adj R-Squared**	0.9662
C.V. %	3.91	**Pred R-Squared**	0.8600
PRESS	292.92	**Adeq Precision**	23.849

Table 9: Standard deviation and correlation coefficients.

Discussion

In the present study, the 23 factorial Central Composite Designs (CCD) of RSM using design expert software (trial version 8.0.7.1, STATE EASE, USA) was applied to optimize the conditions of pH, temperature and substrate concentration. The CCD enables to locate the correct values of temperature, pH and substrate concentration for maximum ethanol production. CCD was successfully used to optimize the key factors that influence the final ethanol concentration in fermentation of corn flour using the SSF method. The main advantages of applying multi factorial experiments are that such an approach considers the interaction between the nonlinear natures of the response in short experiments.

Three design matrixes were made by CCD; thirteen batches for each matrix were run in a 3 L fermenter. The first matrix designs conducted by CCD include combined effect of variables, temperature

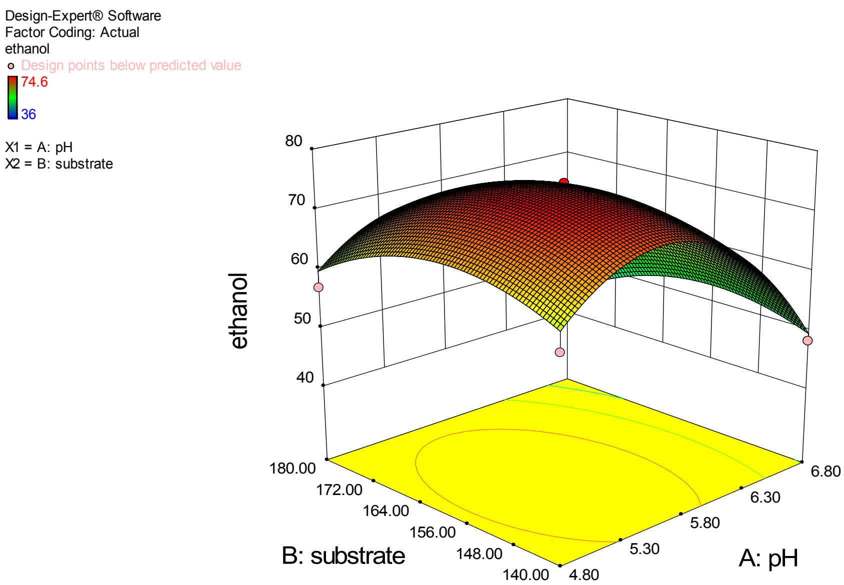

Figure 5: Response surface and contour plot showing the effect of pH and substrate concentration on ethanol production.

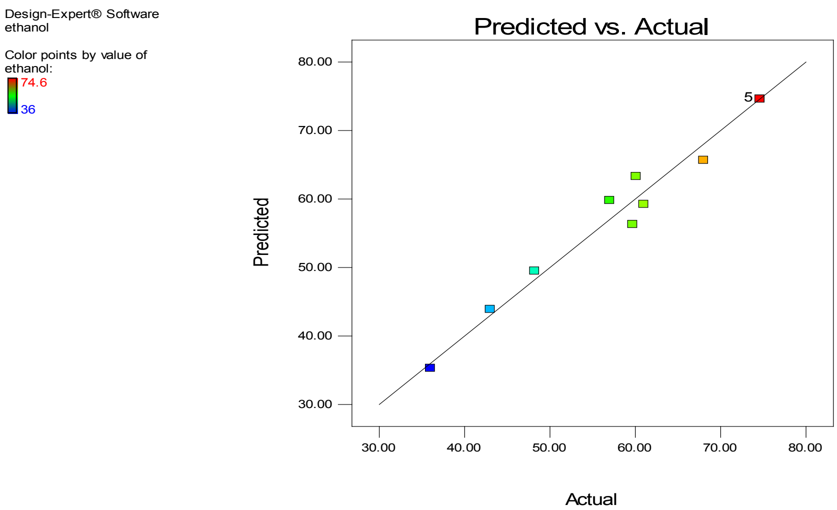

Figure 6: Predicted *vs.* Actual response of experimental run under CCD.

and pH while the substrate concentration remained constant at 160 g. The second matrix design conducted by CCD includes combined effect of variables temperature and substrate concentration at constant pH 5.8. The third matrix design conducted by CCD includes combined effect of variables pH and substrate concentration at constant temperature 31°C. The maximum ethanol production (74.6 g/L) was achieved at combination–pH 5.8, temperature 31°C. However, the minimum ethanol concentration (36 g/L) was obtained at pH 7.21 and temperature 31°C in the first quadratic model. The maximum ethanol production (74.6 g/L) was achieved at temperature 31°C and substrate concentration 160 g. The minimum ethanol production (47 g/L) was found to be at temperature 43.7°C and substrate concentration 160 g in the second quadratic model. The maximum ethanol production (74.6 g/L) in this experiment was found to be at pH 5.8 and substrate concentration 160 g. The minimum ethanol production (36 g/L) was reported at pH 7.21 and substrate concentration 160 g in the third quadratic model.

The microbial culture is highly effective at pH 5.8. At higher pH yeast produces acids rather than alcohol [13,14]. Also at acidic pH there are no chances of bacterial contamination. Yadav et al. [15] found an increase in alcohol concentration, productivity as well as efficiency with an increase in pH from 4.0-5.0 and found that the optimum pH range for *S. cerevisiae* strain HAU-1 to be between pH 4.5-5.0. The temperature was also found to be having profound effect on ethanol concentration. The highest ethanol was observed at a temperature of 31°C. At high temperature there might be the thermal deactivation of enzymes as well as yeast which might be responsible for lesser production of ethanol. Torija et al. [16] observed different responses to fermentation temperatures (15-35°C) on mixed strain population of *S. cerevisiae*. Some strains performed better at higher temperature, while others did so at lower temperature. Alcohol yield was higher at lower temperature while at higher temperature secondary metabolite production increases. It is clearly depicted from the results that substrate concentration also plays a vital role in ethanol production during fermentation process. At high substrate concentration there is lower heat and mass transfer rate during fermentation process which will inhibit the growth of yeast. At high substrate concentration the growth parameters are inhibited due to high medium osmolality [17]. High substrate concentration also causes inefficient fermentation [18]. In the present study, the maximum ethanol production was observed at substrate concentration 160 g/L, followed by 140 g/L and 180 g/L.

In the first quadratic model, the effect of pH is more significant than temperature. In the second quadratic model, the quadratic effects of both the variables were more significant than the linear effects. However, the linear effect of temperature was a slightly more than substrate concentration on ethanol production. The effect of pH was found to be more profound than substrate concentration in the third quadratic model. It can be established that the change in pH has more profound effect on ethanol production followed by temperature and substrate concentration.

The values of adjusted R^2 in all the three quadratic models were high (0.9923), (0.9735) and (0.9662) which are the supporters to high significance of the models. The Coefficient of Variation (CV) indicates the degree of precision with which the treatment is compared. Usually, higher the value of CV, lower is the consistency of the experiment. Here, the values of CV i.e. (2.20%), (2.84%) and (3.91%) indicates the reliability of the experiments performed. Adequate precision is a measure of signal to noise ratio (43.550), (24.310) and (23.849) indicates a better precision and reliability of the experiments carried out. A ratio greater than 4 is desirable. In the present study, the ratio of (43.550), (24.310) and (23.849) indicates an adequate signal to use the models for prediction purposes.

The maximum ethanol production (78 g/L) during the first experiment i.e. by changing one variable at a time was found to be at conditions-pH 5.5 and temperature 35°C. It was found from the statistical analysis of the results that ethanol concentration was effected more by pH followed by substrate and temperature. This is due to the fact that at high pH yeast cannot survive, rather than ethanol it produces acids.

Conclusion

This study concluded that ethanol concentration is greatly affected by the parameters such as pH, temperature and substrate concentration. The outcome of this study has undoubtedly indicated that RSM is an effective method for optimization of fermentation process. RSM confines the number of experimental runs and is less labor intensive. Therefore, smaller and less time consuming experimental designs could generally be sufficient for the optimization of many processes. The adequacy of all the models was satisfactory as correlation coefficients were (0.9923) (0.9735) and (0.9662). The optimum conditions as stated by further numerical analysis of the responses using the Design Expert Software revealed that the maximum ethanol production was achieved at pH 5.8, temperature 31°C and substrate concentration 160 g. The RSM results also depicts that all the models were significant at 99% significance level.

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