Process optimization for ethanol production from very high gravity (VHG) finger millet medium using response surface methodology

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Received: 04 May 2011
Accepted: 02 Jan 2012

Abstract
The Box-Wilson central composite design (CCD) based on response surface methodology (RSM) was used for ethanol fermentation using very high gravity (VHG) finger millet hydrolysate. Optimized process variables were namely, concentrations of yeast extract, magnesium sulphate and pH of the medium. High gravity mashes (>300 g dissolved solids per liter) were prepared by a thermo-stable α-amylase, followed by simultaneous saccharification and fermentation (SSF) at 30ºC for 60 h. Ethanol concentration as high as 13.66% (v/v) was obtained after optimizing the variables. The coefficient of determination (R^2) value of 0.9808 indicates the goodness of fit for regression model. The predicted values for optimization process conditions were in good agreement with experimental data. The optimum values for tested variables were yeast extract 7.13 (g/l) magnesium sulphate 23.32 mM and pH 4.8. Verification of the model indicated no significant difference between predicted and observed values.
Keywords**:** Ethanol production; Finger millet medium; Optimization; Response surface methodology; Very high gravity (VHG) fermentation

INTRODUCTION

Globally, fuel ethanol has become an immediate viable alternative rapidly exhausting fossil fuel deposits, and increasing concerns over environmental pollution. The ethanol market is expected to reach a level equivalent to 10-20% of the gasoline consumption by 2030 (Walter *et al*., 2008). A wide range of substrates can be used as feedstock for ethanol production, including fermentable sugary and starchy substrates to series of steps involved lignocellulosic biomass conversion. The profitability of fuel ethanol production is crucially determined by cost of feedstock used. The feedstock cost typically represents more than 50% of the total production cost, and is the driving factor for researching the potentials of low-cost lignocellulosic biomass for ethanol fermentation (Bai, 2007). In the recent past few years, although a remarkable progress has been made towards development of technology for biomass conversion to ethanol, it is still economically problematic to replace sugar and starch materials in the near-future (Bungay, 2004).

After feedstock costs, energy costs for ethanol fermentation is about 30% of the total production cost. About 80% of the energy consumption is involved in the downstream processing after fermentation, mainly in the distillation of dilute broth as well as in the treatment of large amount of waste stillage by the multiple evaporation technology (Bai, 2007). Therefore, a technology for rapid fermentation and high ethanol concentrations in fermented mashes is desirable; it also helps in decreasing the energy utilization, following cost effective production can be achieved. In recent years, the application of very high gravity (VHG) fer-

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mentation enables doubling of the ethanol content of the fermentor an increase from 7-10 to 15-18% (v/v) (Ingledew, 2005). VHG fermentation is defined in the fuel alcohol context as the preparation and fermentation of media containing 300 g or more dissolved solids per liter (Thomas *et al*., 1993).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of variables and seeking for the optimum conditions. RSM is widely being used in optimization of different types of fermentations and bioprocesses (Kristo *et al*., 2003; Wejse *et al*., 2003; Dey *et al*., 2001). The main advantage of RSM is reduced number of experimental runs needed to provide sufficient information for statistically acceptable results, its suitability for multiple factor experiments and exploration of common relationship between various factors towards finding the most appropriate production conditions for the bioprocess and forecast response (Chang *et al*., 2006). Using a mathematical model, uniform design 0610 (UD 0610), successful optimization of fermentation parameters was carried out for ethanol production by applying corn flour substrate (Wang *et al*., 2007). Supplementation of horse gram flour and finger millet malt to VHG fermenting medium has shown to have a profound effect on yeast viability, thereby promoting increased ethanol yield and decreased fermentation time (Reddy and Reddy, 2006; 2005).

Yeast extract is rich in vitamins especially B complex, amino acids and other growth factors. Magnesium sulphate is one among the key micronutrients for yeast growth and fermentation. Both are essential in yeast nutrition, especially under very high gravity conditions that increased fermentation activity was observed with peptone-yeast extract supplementation (D'Amore *et al*., 1988). Magnesium plays a key role in relieving ethanol toxicity during yeast fermentation (Dombek and Ingram, 1986). The pH of the fermenting medium is another important parameter that needs to be controlled to avoid unwanted bacterial growth, especially lactic acid bacteria and to promote growth of cultured microbes. These factors were found to play a key role in the VHG fermentation of finger millet medium (Pradeep *et al*., 2010). Therefore, the objective of the present study was to optimize the concentration of effective nutrients in VHG ethanol production from finger millet mash by *Saccharomyces bayanus*. RSM is used to optimize the three factors, namely yeast extract, magnesium sulphate and pH of the fermentation medium.

MATERIALS AND METHODS

Finger millet and enzymes: Finger millet (*Eleusine coracana* L.) seeds were procured from a local agricultural market. They were dried and milled to a particle size of 40 BS (British Standard) mesh in an Apex mill. Moisture content of starch flour was determined by AOAC Method 925.10 (Air Oven Method), and was found to be 12 ± 0.38% (dry basis). The starch content of the flour was 67.4 ± 2.89% (dry basis). For starch liquefaction, a heat-stable α-amylase preparation, Biotempase L (Biocon Ltd., Bengaluru, India), having an activity concentration of 1,00,000 BAA units/g and optimal activity in the pH range of 5.5-6.5 was used. For saccharification after liquefaction, Amylo 300 l, a mixture of glucoamylase (260 GAU/g) and pullulanase (390 ASPU/g) was used.

Inoculum preparation: Ethanol tolerant, non-amylolytic and flocculating yeast, *Saccharomyces bayanus* was used in the present study. The culture was preserved at 4°C by regular sub-culturing (once in three months) over MPYD agar (Wickerham, 1951). For inoculum preparation, flasks containing finger millet hydrolysate (10% reducing sugars) supplemented with 1% urea and 2% yeast extract were used and autoclaved (121°C for 15-20 min). After cooling to room temperature, a loopful of cells from a colony on YPD plates was transferred to each flask. Yeast cells were precultured at 30°C on a rotary shaker (130 rpm) for 24 h. The cells were harvested by centrifugation at 2000 ×g for 5 min. The pellet was washed twice with 30 mM/L EDTA to ensure floc disruption and finally washed and suspended in sterile deionized water.

Simultaneous saccharification and fermentation (SSF): Flour slurry was prepared by adding (1:2 ratio) hot tap water (60°C) containing 1mM $CaCl_2$. The pH was adjusted to 6.0 using 1 N HCl, and then 0.4% (v/w) thermo-stable α-amylase was added (Pradeep *et al*., 2010). Both gelatinization and liquefaction processes were allowed to take place in a single step by using an autoclave where its temperature was maintained at 105-110°C for 20 min by regulating the pressure. After liquefaction, un-dissolved solids were removed by filtration through a muslin filter cloth. The mash temperature was cooled to 60°C, pH was adjusted to 4.5. Glucoamylase at a dose of 0.5% (v/w), starch and nutrients such as peptone, yeast extract and magnesium sulphate were added. The enzyme was then allowed to react (pre-saccharification) with liquefied

starch for 1 h at 60°C. Thereafter, the medium was cooled to 30°C, pH adjusted to 5.0 and inoculated with pre-cultured *S. bayanus*. Initial concentration of yeast in the fermenting medium was 2×10^7 cells/ml. Further progression of saccharification along with fermentation was allowed to occur simultaneously for 60 h at 30°C. Each experiment was conducted in triplicate flasks.

Determination of ethanol: Ethanol concentration was determined by gas chromatography (GC) equipped with a flame ionization detector (FID) (Anthony, 1984). Agilent systems model 6890 GC was used under the following conditions: Graphitized packed column 5% carbowax 20 M phase, matrix 80/120 carbopack-B, and Length 6 ft (1.83 m) × 2 mm ID × 1/4-inchOD. Nitrogen at flow rates of 20 ml/min was used as carrier gas. Hydrogen was used as fuel gas, at flow rate 40 ml/min, along with air at a flow rate of 400 ml/min. GC yields determined using sec-butyl alcohol as internal standard

Experimental design: The experimental design and statistical analysis were performed according to the RSM using Design-Expert software (Trial Version 7.1.5, Stat-Ease, Minneapolis, 2008). Central composite experimental design (CCD), with quadratic model (Box and Wilson, 1951) was employed to study the combined effect of three independent variables namely yeast extract (g/l), magnesium sulphate (mM) and pH of the fermenting medium. The dependent variable or response was final ethanol yield % (v/v). In CCD, the range and the levels of the variables investigated in this study are given in the Table 1. The second order polynomial equation (1) was calculated with the statistical package (Stat-Ease Inc, Minneapolis, MN, USA) to estimate the response of the dependent variable. The variance for each factor assessed was partitioned into linear, quadratic and interactive components and were represented using the second order polynomial function as follows:

$$Y= b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{23}x_2x_3 + b_{13}x_1x_3 \quad (1)$$

Where Y is the predicted response variable; x_1, x_2, x_3 are independent variables, b_0 is the 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. The significance of all terms in the polynomial functions were assessed statistically using F-value at a probability (P) of 0.001, 0.01 or 0.05. The regression coefficients were then used to generate contour maps from the regression models. The three-dimensional (3D) plots were generated by keeping one variable constant at the center point and varying the other variables within the experimental range.

Table 1. Actual values of factors used in central composite design.

Factor	Name	Unit	Low actual	High actual
1	Yeast extract	g/l	4.0	12.0
2	$MgSO_4$	mM	10	40
3	pH	--	4.0	5.5

RESULTS

Interactive effects of variables on ethanol production: For optimization of process parameters, statistical experimental design approach was used to provide information on the interactive effect of a few variables, whose impact identified during screening process on response variable. Finally, verification of experiments is used to validate the results under specific experimental conditions (Chen *et al*., 2002). The influence of yeast extract (g/l), magnesium sulphate (mM), and pH on ethanol production was determined using RSM. The results of a three factor, two level factorial experiment designs with five replications of the central point and six axial points are summarized in Table 2. The effect of each factor and their interactions were analyzed using the analysis of variance (ANOVA) and X^2 test as they deem appropriate to the experimental design being used.

Statistical analysis: Central composite design (CCD) is the most common experimental design used in RSM, and the design exhibits equal predictability in all directions from the center. The F-test analysis of variance (ANOVA) was used to check the statistical significance of model equation.

In Table 3, the quadratic model F Value of 28.32 implies that the model is statistically significant. The goodness of the model can be checked by different criteria. Fischer's F-test indicates the overall significance of model; and its associated probability P (F), correlation coefficient R, coefficient of determination R^2 measure the goodness of fit of regression model. In this case, the value of R^2 is 0.9808, indicating that about 1.92% variation is not explained by the model. The R^2 value for the response variable was higher than

Table 2. Central Composite Design (CCD) factors for ethanol production.

Std	Run	(χ_1) A:Yeast extract g/l	(χ_2) B:$MgSO_4$ mM	(χ_3) C:pH --	Response 1 Ethanol concentration (% v/v)	
					Predicted	Experimental
15	1	8.00	25.00	4.75	13.55	12.80
11	2	8.00	25.00	4.75	13.55	13.60
9	3	8.00	25.00	3.69	9.37	9.30
12	4	8.00	25.00	4.75	13.55	13.75
8	5	8.00	46.21	4.75	11.17	11.10
4	6	4.00	10.00	4.00	9.23	9.30
7	7	8.00	3.79	4.75	11.37	11.30
14	8	8.00	25.00	4.75	13.55	13.94
5	9	2.34	25.00	4.75	10.07	10.00
13	10	8.00	25.00	4.75	13.55	13.80
1	11	12.00	40.00	4.00	9.53	9.60
3	12	4.00	40.00	5.50	9.83	9.90
10	13	8.00	25.00	5.81	12.47	12.40
2	14	12.00	10.00	5.50	9.63	9.70
6	15	13.66	25.00	4.75	10.87	10.80

Table 3. Analysis of Variance (ANOVA) table (Partial sum of squares) for Response Surface Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F
Model	44.28	9	4.92	28.32	0.0009**
x_1	0.32	1	0.32	1.84	0.2328
x_2	0.020	1	0.020	0.12	0.7482
x_3	4.80	1	4.80	27.66	0.0033*
x_{12}	18.30	1	18.30	105.31	0.0002**
x_{22}	10.03	1	10.03	57.71	0.0006**
x_{32}	13.34	1	13.34	76.79	0.0003**
x_1x_2	1.70	1	1.70	9.77	0.0261*
x_1x_3	0.077	1	0.077	0.44	0.5360
x_2x_3	0.13	1	0.13	0.77	0.4217
Residual	0.87	5	0.17		
Lack of Fit	0.053	1	0.053	0.26	0.6373
Pure Error					
Cor Total	0.82	4	0.20		
	45.15	14			

*P < 0.05 – significant at 5% level, **P < 0.001 – significant at 1% level

0.90, showing that the regression model explained the reaction well. The value of adjusted R^2 is high (0.9461) so as to advocate high significance of the model. The value of coefficient of variation (CV=3.65) was low due to the small residue between actual and predicted ethanol concentrations. Adequate precision, a measure of signal to noise ratio (12.694) indicates a better precision and reliability of the experiments carried out. A ratio greater than 4 is desirable. In the present case, the ratio of 12.694 indicates an adequate signal to use the model for prediction purposes (Montgomery, 2001). Considering these criteria the response model for alcohol production was:

$$Y = -52.59932 + 1.53683x_1 + 0.016950x_2 + 23.61915x_3 - 0.096250x_1^2 - 5.06667x_2^2 - 2.33778x_3^2 + 0.015350x_1x_2 - 0.065237x_1x_3 + 0.022919\ x_2x_3 \quad (2)$$

Where, Y is the ethanol concentration % (v/v), x_1, x_2,

and x_3 are coded variables.

The response (ethanol concentration) was correlated by non-linear regression using the full quadratic polynomial model. Values of probability >F indicate that model terms are significant. In this case x_3, x_1^2, x_2^2, x_3^2, and x_1x_2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Though the variables x_1 (Yeast extract) and x_2 ($MgSO_4$) are insignificant in the linear terms, they are more significant (P<0.001) in quadratic terms (i.e., the factors have a more influence on the production of alcohol and changes in those variables will significantly affect the process) and they are very important nutrients in yeast ethanol fermentation. Further more, Table 3 shows that the interaction effect of these two variables plays a prominent role in the enhanced yield of ethanol, statistically significant at P<0.05. The "Lack of Fit F-Value" of 0.26 implied that the lack of fit is not significant relative to the pure error (Table 3). The analysis of the variance (ANOVA) of the quadratic regression model demonstrated that equation (1) is highly statistically significant predictor of ethanol concentration, was evident from the Fisher's F-test with a very low probability value (0.0009).

Optimization of ethanol production: By using response surface 3D plot, the interaction between two variable factors and their optimum levels could be easily understood. The Figure 1 shows the effect of yeast extract and pH on ethanol concentration (% v/v) with other variables at zero level. Maximum ethanol concentration was observed in the pH range of 4.75 to 5.1, and yeast extract concentration near the central value of 8.0 g/l. The Figure 2 exhibits the interactive effect of magnesium sulphate and pH on ethanol concentration with other variables at zero level. As shown in the plot, optimal ethanol concentration was observed under increased pH from central value of experimented range, near to 5.0, and 25 mM of $MgSO_4$. Whereas in the case of yeast extract and magnesium sulphate interaction, as shown in Figure 3, concentrations near mid-points were observed as optimal values. A verification run was conducted in two replicates to confirm the optimal condition. As depicted in the Figure 4, the parity plot showed a good correlation between the experimental values and predicted values, wherein the data points distributed along the diagonal line, which indicates the good fit of the model.

For selection of the optimum conditions and range, the model was analyzed separately. From the validation report, the required criteria were selected with maximum ethanol as the target. Choice of solutions was automatically retrieved by the software. The target goal was to reach maximum ethanol yield with limited yeast extract supplementation as yeast extract is one of the costly nutrient sources for fermentation. So, minimum yeast extract was selected. The levels of $MgSO_4$ were selected in range of 10-40 mM. The pH was also in the range of 4-4.8. The pH beyond 5.0 is generally undesirable in ethanol fermentations, which is likely due to bacterial contamination. Ethanol target is above 13.5%; lower limit is 13% and upper limit is 13.9% (v/v).

The maximum response predicted from the model was 13.5 g/l (Table 4). Repeated experiments were performed to verify the predicted optimum. The result from three replications was coincident with the predicted value; the average was 13.66 g/l and the model

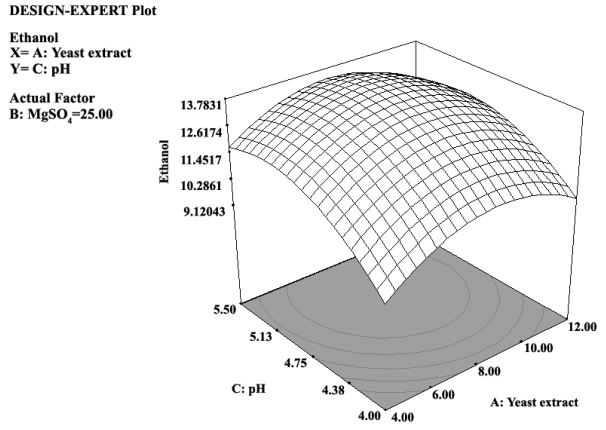

Figure 1. Response surface and contour graphs shows the effect of yeast extract and pH on ethanol concentration % (v/v) with other variables at zero level.

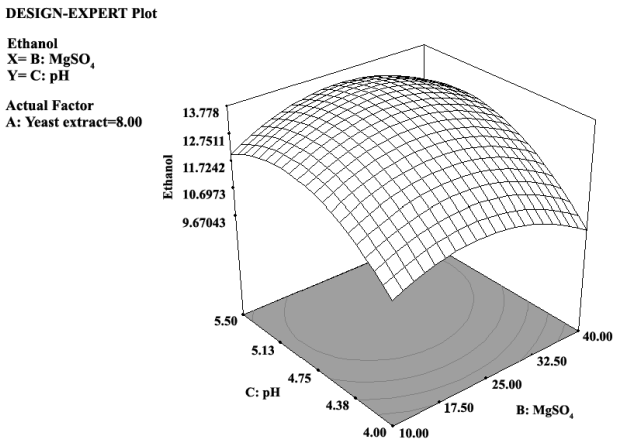

Figure 2. Response surface and contour graphs shows the effect of magnesium sulphate and pH on ethanol concentration % (v/v) with other variables at zero level.

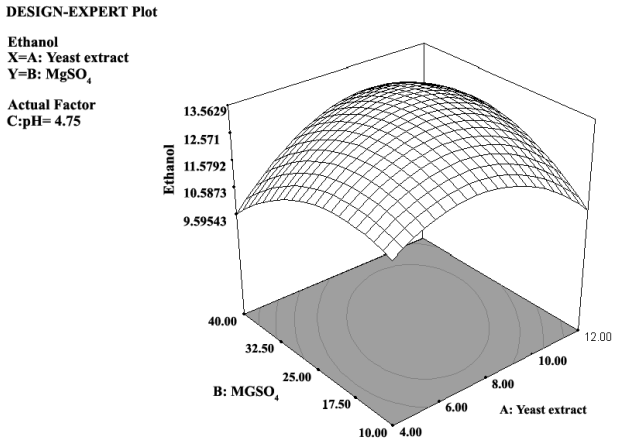

Figure 3. Response surface and contour graphs shows the effect of yeast extract and magnesium sulphate on ethanol concentration % (v/v) with other variables at zero level.

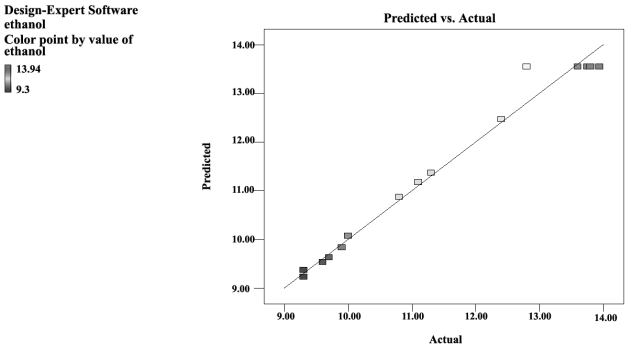

Figure 4. Parity plot shows the distribution of experimental *vs.* predicted values of ethanol production.

proven to be adequate. The final optimized fermentation conditions obtained with RSM were 7.13 g/l of yeast extract, 23.32 mM of $MgSO_4$ and a pH of 4.8. Compared to un-optimized medium, nearly 20% increase in final ethanol yield was observed under statistically optimized conditions.

DISCUSSION

The 2^3 factorial central composite design (CCD) was applied to optimize the conditions of enzymatic saccharification of food waste, and thereafter ethanol fermentation (Kim *et al*., 2008). The CCD enables to find the accurate values of concentration of medium constituents, namely concentration of sugar, nitrogen, EDTA and fermentation conditions (temperature, pH and time of fermentation) for ethanol production using palmyra jaggery (Ratnam *et al*., 2005). CCD was successfully used to optimize the key factors that influence the final ethanol concentration in very high gravity fermentation of cassava mash using the SSF method (Yingling *et al*., 2011). Importance of Mg^{2+} and yeast extract under VHG conditions has been detailed in an earlier report (Wang *et al*., 2007); wherein over 20% increase in final ethanol concentration was observed through optimization by a uniform design process, using corn hydrolysate substrate. In our previous report, an ethanol concentration of 15.6% (v/v) was reported by separate hydrolysis and fermentation (SHF) process (Pradeep *et al*., 2010) in about 72 h fermentation time excluding pre-saccharification time. However, in the present study, through establishing optimized conditions, pre-saccharification time prior to fermentation has been reduced from 24 to 1 h under SSF conditions compared to previous SHF study. Therefore, significant processing time and corresponding energy costs savings can be achieved, though the final ethanol yield was slightly decreased in SSF process. As the results confirm, through statistical optimization, expensive nutrients such as yeast extract and mineral salts can be used effectively with minimal waste, and thereby reduce the overall cost of the process.

The process of ethanol fermentation using finger millet is viable because unlike ethanol from wheat or corn, this feedstock do not compete with food-products or land-for-food. Also, finger millet is a rich source of carbohydrate content (average of 60%

Table 4. Optimum values of fermentation variables, experimental and predicted ethanol yields.

Variables	Optimum values	Optimal ethanol yield (g/l)	
		Experimental	Predicted
Yeast extract (g/l)	7.13	13.66±0.25	13.5
$MgSO_4$ (mM)	23.32	-	-
pH	4.8	-	-

Number of samples n=3.

starch); drought-tolerant, and therefore, ideal crop in dry areas. Furthermore, unlike other starches, finger millet can be stored for long periods without insect damage and thus surplus production can be conveniently processed to high-value products like fuel ethanol industrially. Moreover, in Africa, especially in Uganda, finger millet is used in the production of fermented beverages like malwa (ajon) and bushera (Steinkraus, 1996). In this study, we exploit the full potential of important but underutilized source of starch, the finger millet, as a feedstock for ethanol production.

CONCLUSIONS

The information generated during this study could benefit existing cereal based fuel alcohol plants without alteration of plant equipment or process flow. The final ethanol concentration close to the predicted value was obtained by the process of optimization. The results of this study have clearly indicated that RSM is an effective method for optimization of fermentation process.

Acknowledgements

We are thankful to UGC (New Delhi), for the financial assistance. Our special thanks to Dr.S.C.Basappa, former Deputy Director (CFTRI, Mysore) and Dr.Y.Sudheer Kumar (IICT, Hyderabad) and for their critical comments on the manuscript.

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